Arthropod-borne infections in the United Kingdom and Saudi Arabia

Bassam Alharbi

Supervised by:

Dr. Kevin Bown

School of Environment and Life Sciences

University of Salford,

Salford, UK

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Declaration.

No portion of this work referred to in this report has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Acknowledgment.

I would like to thank Allah for giving me this opportunity to complete this thesis. Furthermore, I would like to thank Dr. Kevin Bown and Professor Richard Birtles for their valuable support, motivation and help, without their guidance this thesis would not be completed. I would also thank my lab mates Jess, Raed, Adam, Andrea and Lauren for co-operation and interaction in the lab. I would like to thank Dr. Abdulaziz Alagaili for his contribution by providing samples that were collected from Saudi Arabia. Finally, I would like to give special thanks to my family for their vital support.

Abstract.

Arthropod vectors can transmit different diseases that are both a significant and widespread cause of mortality and morbidity in both human and wildlife species. However, further studies are required to identify the role of wildlife species as reservoir hosts for such infections. In some countries, such as Saudi Arabia, few studies have taken place whist in other regions individual species may have been neglected, such as red foxes (*Vulpes vulpes*) in the United Kingdom (UK).To address this paucity of knowledge, samples from different host species from the UK and Ireland (small rodents, red foxes, shrew and ticks species) and from Saudi Arabia Libyan jirds (*Meriones libycus*) and desert hedgehogs (*Paraechinus aethiopicus*) were collected and screened for different haemoparasites including *Trypanosoma* spp., *Babesia/Theileria* spp., and *Bartonella* spp. The data within this study describe different haemoparasite prevalences from these different hosts using PCR- based molecular typing tools. *Trypanosoma* infections were found in mall rodents from the UK and Ireland, however the presence of the invading bank vole (*Myodes glareolus*) in Ireland appears to have disrupted the host-parasite relationship between wood mice (*Apodemus sylvaticus*) and trypansomes. *Babesia vulpes* was identified in 134/392 (34%) of red foxes in the UK, suggesting that this potentially important parasite may be common in the UK. From Saudi Arabia, the data within this study showed that 49/121 (40%) of jirds were infected with *Theileria* spp. whereas 74/112 (66%) of hedgehogs harboured this parasite. Furthermore, *Bartonella* spp. infections were found in both jirds and hedgehogs from Saudi Arabia, where 73(60% of jirds and 15(13%) of hedgehogs were found to be infected with *Bartonella*.

To compare the methods of PCR, real- time PCR and the newest technique, Next Generation Sequencing (NGS), a number of *Ixodes ricinus* tick samples were screened by NGS by analysing 16S rRNA gene and the resultant data were confirmed by either PCR or real-time PCR. Different bacterial infections were found in the samples including *Anaplasma phagocytophilum*, *Borrelia graini*, *Candidatus Midichloria,* and *Rickettsia Helvetica*. The comparison between these techniques revealed that NGS offers the potential to be a useful tool in screening hosts and vectors for infections, particularly in identifying novel infections.

Chapter One

Introduction and the aims of this thesis

1. Introduction.

Diseases transmitted by arthropod vectors are a significant cause of mortality and morbidity, with one sixth of the world's population estimated to be at risk of infection (WHO, 2014). Indeed it is estimated that as many as one billion people are infected and more than one million deaths are caused each year by vector-borne illnesses (WHO, 2014). Malaria alone was responsible for an estimated 627000 deaths in 2012 (WHO, 2014). In addition to human disease, vector-borne infections are important from a veterinary perspective, with examples of *Babesia* infection in cattle. The importance of such infections demands that effective control strategies can be developed, something that requires an in-depth knowledge of the infection's ecology and epidemiology. Many arthropod-borne infections circulate in wildlife, such as *Trypanosoma* spp, *Babesia* spp and *Bartonella* spp. As such, the study of disease in wildlife species can provide insights into infections of medical and veterinary importance. Transmission of haemoparasitic diseases by arthropod species is a complex phenomenon that has been observed worldwide (Goddard, 2000). Invertebrates of this phylum, which are capable of acting as competent vectors include mosquitoes, spiders, lice, fleas, and ticks. These are the most important Arthropoda species for transmission of various pathogens to a vertebrate host. Transmission of pathogens including protozoa, viruses, bacteria, and helminths to humans results in a variety of clinically important human diseases, such as Lyme disease, malaria, babesiosis, bartonellosis, yellow fever and theileriosis (Hill *et al.*, 2005).These infections result in significant morbidity and mortality across the globe with many of these being resistant to chemotherapy or lacking a plausible prophylactic vaccine. Transmission of haemoparasites to wild animal reservoirs is also an important phenomenon since these may act as intermediate hosts between vector and humans. This has been evidenced in several studies especially for those individuals who may come into direct contact with wild animals and their vectors (Lempereur *et al.*, 2012; Tokarska-Rodak *et al.*, 2016), especially in relation to *Borrelia* spp and *Babesia* spp. In this study different haemoparasite infections have been investigated from different host species that were sampled in the United Kingdom and Saudi Arabia.

1.2. Protozoan parasites.

1.2.1 .Trypanosomiasis.

The family Trypanosomatidae is a group of protozoan parasites belonging to the order Kinetoplastida, which is believed to have an ancient eukaryotic lineage (Sogin *et al.*, 1986). The genus *Trypanosoma* is widespread, consisting of blood parasites that can infect all classes of vertebrates, causing several human and livestock diseases, especially in the tropics. Currently, more than 470 species are considered members of the *Trypanosoma* genus. *Trypanosoma* species are divided into two main groups, Salivaria and Stercoraria, according to their main transmission mechanism (Haag *et al.*, 1998). The most representative example for Stercoraria is *Trypanosoma cruzi,* which completes its life cycle in the posterior section of insects. On the other hand, species from the Salivaria group complete their life cycle within the anterior section, which is the case for *T. brucei* (Alvarez *et al.*, 1996).

1.2.1.1. Host range of *Trypanosoma*.

Different mammal species act as reservoir hosts for *Trypanosoma* spp, maintaining these parasites in the environment (Table 1.1).

Table1.1. Examples of *Trypanosoma* host-vector relationships.

***Trypanosoma* species**	**Host**	**Disease**	**Vector**	**Distribution**	**References**
T. b. gambiense	Humans and domestic animals	Sleeping sickness	Tsetse fly	West and Central Africa	(Steverding, 2008)
T. b. rhodesiense	Humans and domestic animals	Sleeping sickness	Tsetse fly	East Africa	(Cox *et al.*, 2005)
T. congolense	Cattle and domestic animals	Nagana	Tsetse fly	Tropical Africa	(Steverding, 2008)
T. cruzi	Humans, domestic and wild animals	Chagas disease	Reduviid bugs	South America	(Rassi & Marin-Neto, 2010)
T. lewisi	Rats	Trypanosomiasis	Fleas	Worldwide	(Molyneux, 1969a)
T. vivax	Cattle, sheep, goat and camel	Nagana	Tsetse fly	Africa and South America	(Dagnachew & Bezie, 2015)

1.2.1.2. Importance of *Trypanosomes*.

Trypanosomiasis is caused by several species belonging to the *Trypanosoma* genus. The disease is caused by blood-borne parasites that can infect vertebrates, including both humans and animals (Hamilton *et al.*, 2004). For instance, human African trypanosomiasis (HAT), also known as sleeping sickness, is as a result of infection with parasites of the species *T. brucei* (Barrett *et al.*, 2003; Brun *et al.*, 2010).This disease

causes neurological disturbances, associated with the second phase of the infection, when the central nervous system is infested by the parasites (Barrett, 1999; Kennedy, 2006).

Infection of humans and animals has become a major concern due to the negative health impacts associated with the disease, also resulting in serious economic losses (Kristjanson *et al.*, 1999). For example, economic losses due to *T. congolense* have been estimated at between US $1.0 to 1.2 billion in cattle production in Sub-Saharan Africa alone (Daffa *et al.*, 2013)

Human African trypanosomiasis is a serious public health problem caused by protozoan parasites, transmitted to humans by tsetse flies (*Glossina* genus) that have fed on infected animals or humans, and harbours the parasite in its infective stage. While HAT may be perceived as being less important than other diseases such as malaria and AIDS worldwide, it is responsible for a high degree of mortality and morbidity in regions where it is endemic (Hide, 1999).However, many species of trypanosome can infect animals and humans. Only thsese spcies can infect human*Trypanosoma brucei gambesiense* and *T. brucei rhodesiense* are responsible for human infection, and *T. cruzi* is the causative agent of Chagas disease in the Americas (Barrett *et al.*, 2003) . Human African trypanosomiasis has been recognised in 36 Sub-Saharan African countries, with about 6 million people at serious risk of disease (Brun *et al.*, 2010).

Furthermore , Chagas disease first detected by Carlos Chagas in 1909, and it is unfortunately still considered a neglected disease despite its massive impact upon those who live in endemic areas (WHO, 2015). This disease has been reported in 22 Latin American countries (Coura & Viñas, 2010), and it was estimated that 8 million people have the disease, while more than 25 million people were at risk of being infected (WHO, 2015). In addition, more than 10,000 people die from Chagas disease every year (WHO, 2015). Chagas disease is a zoonotic disease, which occurs in wild animal species and can be transmitted to humans (Coura & Viñas, 2010). The disease is mostly confined to Central and South America, however, with increasing immigration, the disease has been reported in the southern parts of North America (Rassi & Marin-Neto, 2010) .

1.2.1.3. Transmission of human African trypanosomiasis and Chagas disease.

Human African trypanosomiasis caused by *T. b. gambiense* is considered an infectious disease that includes a minor role of animal reservoirs, whereas HAT caused by *T. b. rhodesiense* is a zoonotic disease that mainly affects animal species, with humans considered an accidental host (Franco *et al.*, 2014). However, it is very rare that animals play important roles in the transmission of *T. b. gambiense* to humans. Njiokou et al (2010) reported the presence of *T. b. gambiense* in domestic and wild animals, and demonstrated the *T. b. gambiense* transmission cycle. Furthermore, some experimental studies have shown that *T. b. gambiense* can only survive for a limited time in animals, lasting less than 1 year (Schütt & Mehlitz, 1981). Further studies are required to understand and identify the role played by animal reservoirs in maintaining the parasites.

The transmission of Chagas disease is a blood-sucking insect known as the triatomine ('kissing') bug. These bugs are active through the night, when the host species (humans and animals) are sleeping. The *T. cruzi* parasite is transmitted through the faeces of the vector, which enters the host's circulatory system when a blood meal is taken by an infected vector. For example, the parasite can reach the bloodstream of the host when they scratch a bite from the vector via any open wounds on the hands, feet or other parts of the body. When in the bloodstream, the parasite starts to reproduce within different organs (Rassi & Marin-Neto, 2010), shown in Figure 1.1.

1.2.1.4. The life cycle of *Trypanosoma cruzi*.

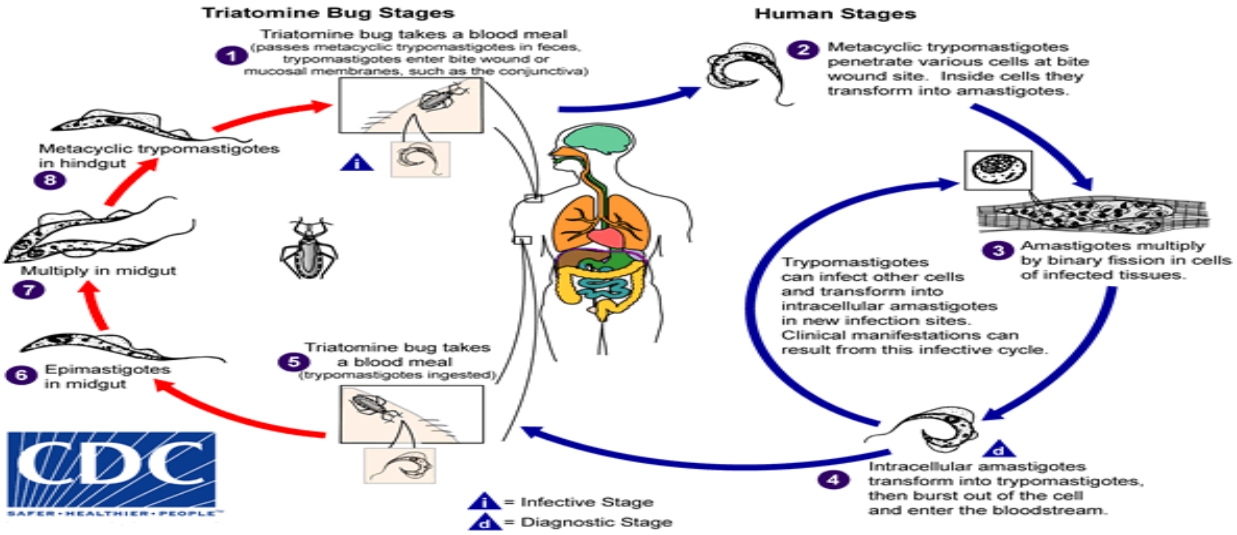


Figure 1.1.The life cycle of *Trypanosoma cruzi* (CDC, 2014). 1:The blood meal is taken by an infected vector and the metacyclic stage enters the body.2: The metacyclic stage invades different cells of the body and transforms into amastigotes forms.3: The amastigote forms multiply in the infected cell by binary fission.4: The intracellular amastigotes transform into trypomastigotes which occur in the bloodstream. 5: The trypomastigotes are taken by the triatomine bug from the infected host.6: In the gut, the trypomastigotes transform into epimastigotes. 7: The epimastigotes multiply inside the midgut.8: The epimastigotes then differentiate into metacyclic trypomastigotes in the hindgut.

Once a blood meal has been taken by an infected triatomine insect, the trypomastigotes released into the faeces near the biting site invade cells close to the site of inoculation. Here they transform into amastigotes, which multiply by binary fission and differentiate into trypomastigotes, which are then subsequently released into the bloodstream. The kissing bugs become infected by feeding on an infected host. Trypomastigotes are ingested and transform into their epimastigote form in the midgut. The parasites multiply in the midgut, and transform into metacyclic trypomastigotes in the hindgut (CDC, 2014).

1.2.1.5. Animal Trypanosomiasis.

As demonstrated, several species of *Trypanosoma* can cause serious disease in domestic and wild animal species in different parts of the world. For example, African animal trypanosomiasis is a parasitic infection associated with serious economic losses in different livestock species. The most important *Trypanosoma* species in Africa are *T. congolense*, *T. vivax*, *T. brucei brucei*, *T. simae* and *T. suis* (Hoare, 1972). These species cause Nagana disease, which can affect the meat and milk production of various cattle species, and has been estimated to contribute to about $1340 million in economic losses every year(Kristjanson *et al.*, 1999), in a continent that consistently struggles financially.

1.2.1.6. Important trypanosomes in the United Kingdom.

Mammalian species in the UK have been demonstrated to be infected with several species of trypanosomes (Lizundia *et al.*, 2011). For instance, British cattle and sheep have been found to be infected by *T. theleria* and *T. melophagium*, respectively. Both of these species belong to the subgenus *Megatrypanum*, and are transmitted by the Tabanid fly and sheep ked (Böse & Heister, 1993; Hoare, 1972). In addition Lizundia et al (2011) detected *T. pestani* in British badgers, with fleas (*Paraceras melis*) identified as the main vector for transmission.

The subgenus *Trypanosoma* (*Herpetosoma*) has been reported in the UK. This subgenus includes several species of rodent parasites, which appear to be morphologically similar to *T. lewisi*, a typical trypanosome found in rats (Dobigny *et al.*, 2011). Despite *T. lewisi* being considered specific for rats, and non-pathogenic for both rats and humans, eight cases of human infection have been reported to date (Tang *et al.*, 2012). One of these cases was a 37-day-old child from India. The mother initially noticed three red spots on the child's leg, associated with subsequent development of inflammation (approximately 2 cm in diameter) surrounding the area of the marks. The child developed new symptoms over the next day, including fever, listlessness and a loss of appetite. A blood sample was analysed using polymerase chain reaction (PCR), and *T. lewisi* was identified as the causative agent of the disease (Verma *et al.*, 2011).

Several studies from the UK have shown that species from the *Trypanosoma (Herpetosoma)* subgenus are commonly found in species such as the bank vole (*Myodes glareolus*) and the wood mouse (*Apodemus sylvaticus*). These studies have also suggested

that fleas are the most likely vector of these infections (Sato *et al.*, 2005; Turner, 1986). *Trypanosome evotomys* is transmitted mainly by *Ctenophthalmus agyrtes* and *Malareus penicilliger*, while *T. grosi* is transmitted by *Ctenophthalmus nobilis* and *C. congerer* (Karbowiak & Sinski, 1996). The distribution of *Trypanosoma (Herpetosoma)* species across Europe remains poorly understood, and it was not until the beginning of the 20th century that trypanosomes in European rodents were first described (Laveran & Pettit, 1909) . The subgenus *Herpetosoma* includes approximately 90 different species and strains (Karbowiak & Sinski, 1996). Most *Trypanosoma* (*Herpetosoma)* species infect rodents from the *Muridiae* and *Microtidae* families (Karbowiak & Sinski, 1996). These parasites seem to infect only one host species and do not have the ability to infect multiple hosts (Guan *et al.*, 2011).

Trypanosoma (*Herpetosoma*) species share common morphological features and hosts. These common characteristics are sufficient to distinguish species from different groups, although morphology itself is not adequate for differentiating species within groups (Guan *et al.*, 2011; Noyes *et al.*, 2002). *Trypanosoma evotomys* and *T. grosi* are morphologically similar, however, other features can be used to identify the different parasites. For example, reproductive phase characteristics have been confirmed to be useful for differentiating the group containing *T. evotomys* and *T. microti* from that of *T. grosi, T. lewisi* and *T. musculi* (Hoare, 1936; Karbowiak & Sinski, 1996; Molyneux, 1970) .*Trypasonoma (Herpetosoma)* species that infect rodents of the Murinae subfamily (*T. grosi, T. lewisi* and *T. musculi*) can be distinguished by their epimastigote phase, which occurs in the peripheral blood, whereas those that infect Microtinae rodents (*T. evotomys* and *T. microti*) can be distinguished by the amastigote phase, as they reproduce in the lymphoid tissue; amastigotes are never observed in the peripheral blood (Table 1.2.) (Molyneux, 1970).

Table 1. 2. Characteristics of *Trypanosoma lewisi* like species (Molyneux, 1970).

Species	Host	Method of reproduction	Main site of reproduction	Dividing form present in blood	Incubation period	Duration of infection	Flea species	Site of development in flea
T. lewisi	(Murinae) Rat	Unequal multiple fission of epimastigote	Kidney	Yes	3–7 days	2–4 weeks	*Nosopsyllus fasciatus*	Rectum
T. musculi	(Murinae) House mouse	Unequal multiple fission of epimastigote	unknown	Yes	3–7 days	30–40 days Or longer	unknown	Rectum
T. grosi	(Murinae) *Apodemus sylvaticus*	Unequal multiple fission of epimastigote	unknown	Yes	7–10 days	60 days or 5 months	*Ctenrophthalamus agyrtes*	Rectum
T.	(Microtina)	Multiple	Lymphoid	No				Pyloric region

evotmys	*Myodes glareolus*	binary fission of amastigote	tissue		5–6 days	22–31 days	*N. fasciatus*	and intestine
T. microti	(Microtina) *Microtus agrestis*	Multiple binary fission of amastigote	Lymphoid tissue	No	6–9 days	23–74 days	*N. fasciatus*	Pyloric region and intestine

1.2.2.*Theileria*.

The *Theileria* genus belongs to the phylum Apicomplexa, which consists of several obligate parasites of vertebrates and invertebrates. These parasites share characteristic features when they start to invade the mammalian or invertebrate host. The Apicomplexa phylum is divided into four groups, the Haemosporidia, Coccidia, Gregarnusina and Piroplasmida (Adl *et al.*, 2012). The Piroplasmida subgroup has two genera, *Babesia* spp and *Theileria spp*, which can infect both domestic and wild animals. Several new species of the Piroplasmida group are still being discovered, and many of these parasites are classified based on the morphological features of these parasites in the red blood cells of the infected host, in addition to their host-vector specificity (Barnett, 1977; Uilenberg, 2006). The parasites can infect a wide range of wild and domestic animals throughout the world. These parasites carry out their reproductive cycle (schizogenous) in the lymphocytes of the host, and usually have a piroplasm stage in the erythrocytes (Dolan, 1989).

Bovine theileriosis is one of the most prevalent diseases in cattle and buffalo populations around the world, associated with significant economic impacts due to the high rate of mortality and morbidity in these species (Forsyth *et al.*, 1997) . The infection can occur in different parts of the world, including tropical and subtropical regions, which are known to be suitable locations for their tick vectors (Khan *et al.*, 2004; Perera *et al.*, 2013).

Mediterranean coast fever, or tropical theileriosis, is considered a fatal disease that infects cattle populations (Santos *et al.*, 2013). This disease is caused by *Theileria annulata*, and can lead to significant production losses (Gharbi *et al.*, 2011). Another economically important parasitic infection is East coast fever, caused by *T. parva* (Gharbi *et al.*, 2011). This parasite is estimated to cause mortality in about 1 million cattle per year in different parts of Africa (Salih *et al.*, 2007). The first description of *T. annulata* was in Transcaucasian cattle in 1904, and another important species, *T. parva*, was first described in 1901 in Zimbabwe and the parasite spread throughout regions in Tanzania and Kenya in which cattle had been imported (Gul *et al.*, 2015) . In 1914, the infection was reported to cause mortality in 1.25 million out of 4 million cattle in infected regions. It was also reported in Mozambique, Zambia and Malawi between 1912 and 1922, and is still found in these countries where it is associated with significant losses to the livestock community (Yusufmia *et al.*, 2010).

The most important tick species contributing to the transmission of tropical theileriosis are from the genus *Rhipicephalus*, which is prevalent in regions including Italy, Spain, Portugal, Turkey, Greece, Asia and the Middle East (Ali & Radwan, 2011; Gul *et al.*, 2015). East coast fever has been reported in different countries in Africa, including Malawi, Sudan, Kenya, Zaire, Uganda, Burundi, Zimbabwe and Tanzania (Gachohi *et al.*, 2012).

Theileria species can infect both wild and domestic animals, transmitted by *Ixodid* tick species such as *Rhipicephalus*, *Haemaphysalis* and *Hyalomma* (Table 1.3). The expansion of wild and domestic animals has made *Theileria* infections an important study area (Mans *et al.*, 2015).

Table 1.3**.** Several *Theileria* species with animal hosts.

***Theileria* species**	**Host**	**Vector**	**Reference**
T. parva	Cattle and African buffalo	*Rhipicephlalus*	(Gul *et al.*, 2015)
T. lestoquardi	Goat and sheep	*Hylomma* spp.	(Bishop *et al.*, 2004)
T. annulata	Cattle and buffalo	*Hylomma*	(Gul *et al.*, 2015)
T. annae	Dog and fox	*Ixodes* spp. and *Rhipicephlalus*	(Zahler *et al.*, 2000)
T .equi	Horse, donkeys and giraffes	*Hylomma* spp. and *Rhipicephlalus*	(De Waal & Van Heerden, 1994)
T. ovis	Sheep and goat	*Hylomma* spp.	(Nagore *et al.*, 2004)

1.2.2.1. *Theileria* life cycle.

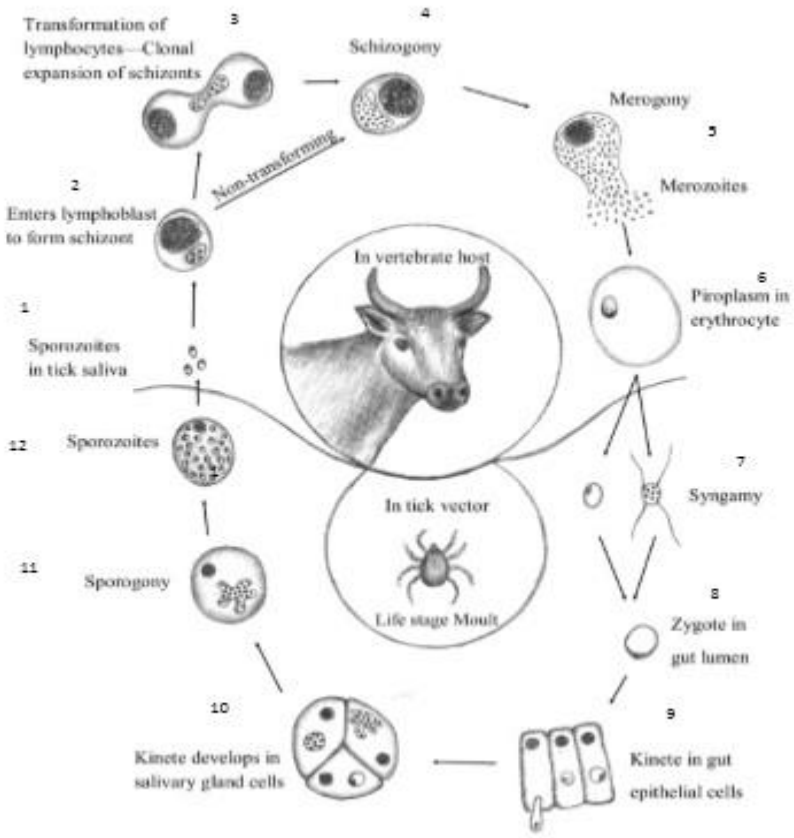


Figure 1.2. The life cycle of *Theileria* species (Mans ,2015).

Sporozoites (1) enter the body and invade the lymphoblast before transforming into schizonts, (2) which multiply in the cell (3) and then undergo the process of schizogony (4). After that, the parasites experience merogony (5) , where infected cells then rapture and the merozoites (6) infect other cells. Once the blood meal is ingested from the infected host, the syngamy occurs and parasites form the zygote (8) in the gut. After sexual multiplication, the zygotes form the kinete (9) which invades the epithelial cell. Once the blood meal is taken, the kinete invades the salivary gland (10) and undergoes sporogony (11) before the sporozoites (12) are formed.

All *Theileria* species have the same life cycle, which has been extensively studied (Barnett, 1968). The parasites develop within the vertebrate and invertebrate host, with asexual reproduction taking place in the vertebrate host and sexual reproduction occurring in the invertebrate host.

The normal transmission of the parasite occurs when infected ticks feed on mammalian hosts, injecting the sporozoites into the body. The sporozoites enter the lymphoid cells of the host (Shaw & Young, 1995) , and sporozoites of different *Theileria* species can invade different sub-types of leukocytes. For example, the sporozoites of *T. parva* are able to invade and survive in all subtypes of lymphocytes (Morrison *et al.*, 1996). Once the parasite is inside the leukocyte, they develop into macroschizonts and induce transformation and proliferation of the cell, resulting in the existence many colonies of parasitized cells (Williams & Dobbelaere, 1993). The parasites divide to synchrony and schizonts appear when the host cell enters mitosis. Merogony occurs with infected lymphocytes (Michael Shaw & Tilney, 1992), followed by rupture of the host cell and subsequent release of merozoites, which subsequently invade red blood cells (Glascodine *et al.*, 1990).

Ticks become infected when feeding on an infected host. The parasites start to develop rey bodies in the gut 2–4 days after feeding (Mehlhorn & Schein, 1985), which form gametes. Syngamy of the gametes takes place in the gut on day 6 after feeding, followed by formation of the zygote. The zygote invades the epithelial cells of the gut then develops a motile kinete which can penetrate the gut and occur in the haemolymph then reach the salivary gland. Sporogony occurs when inside the epithelial cells of the salivary gland, and this stage is completed when the tick moults and starts to feed on a new host. The sporozoites are formed and inoculated though the skin of the host (Billiouw, 2005).

1.2.2.2. Clinical manifestations of Theileriosis.

Clinical symptoms induced by several *Theileria* species can result from massive lymphocyte eradication, which occurs when the parasites multiply inside the host. This results in cell rupture. Therefore, the severity of theileriosis symptoms can vary, including fever, anorexia, decreased milk production, enlargement of lymph nodes or lymphoid

tissue, and infiltration of lymphoid into other body organ such as the kidney (El-Deeb & Younis, 2009).

1.2.3. *Babesia*.

Babesiosis is a tick-borne zoonotic disease of both humans and domestic animals, caused by protozoan parasites of the genus *Babesia* and phylum Apicomplexa. There are more than 100 *Babesia* species, which can infect various mammalian species, and they are considered to be the second most widespread blood parasites worldwide after trypanosomes (Telford, Gorenflot, Brasseur, & Spielman, 1993). These pathogens can infect many vertebrates such as carnivores, rodents and cattle (Hunfeld, Hildebrandt, & Gray, 2008). Currently, molecular analysis is used to classify *Babesia* species, rather than older methods based on host specificity and morphological parameters. There are several problems associated with the older diagnostic methods, including different species of parasites having a similar appearance to the same host, pathogens having a different macroscopic appearance due to host-specific factors, and the host specificity of *Babesia* species appears to be less useful for classification due to the broad range of hosts infected by *Babesia* species, such as *B. microti* (Brandt *et al.*, 1977; Moore & Kuntz, 1981).

Babesia species are transmitted by *Ixodid* ticks to the vertebrate host, where they replicate in the red blood cells (Homer *et al.*, 2000). More than six species of *Ixodid* ticks are considered to be the main vectors of *Babesia* species (Schein *et al.*, 1981). Some of these species, including *B. bigemmina*, are able to infect more than one genus of ticks. On the other hand, some species (*B. microti*) can only infect species of the *Ixodes* genus (Telford et al., 1993) . Furthermore, a study by Najm et al. (2014) reported the presence of different species of *Babesia*, such as *B. microti-like* species (also known as *Theileria annae*), in three tick species, *Ixodes canisuga, I. hexagonus* and *I. ricinus*. While *I. hexagonus* is absent in South Korea and the USA, *B. microti-like* species *(T. annae*) has been detected in these areas. Thus, this provides strong evidence that other tick species play an important role in the transmission of this disease in these regions (Han *et al.*, 2010). Recently, the parasites have been isolated from 46/316 (14.6%) red foxes in the red fox population in the UK (Bartley *et al.*, 2016). However, no studies have been done to investigate the presence of *T. annae* in different tick species.

1.2.3.1. Human Babesiosis.

Human babesiosis is caused by several *Babesia* species, which have a distinct geographical distribution, depending on the presence of the animal species. For example, more than 29 cases of humans infected with *Babesia divergens* have been reported in Europe, and many of the patients reported a history of tick bites, which appears to be the main route of infection (Gorenflot *et al.*, 1998). The increasing evidence of human babesiosis in Europe has been attributed to the expanded cattle population, with largest increase in incidence occurring between May and September when tick species are most active (Gorenflot *et al.*, 1998). *Babesia divergens* is considered to be a bovine parasite transmitted by *I. ricinus*, which is thought to be responsible for several cases of human babesiosis. The potential risk of the disease is becoming of greater public health concern than previously thought, due to the wide range of reservoir hosts (Chauvin *et al.*, 2009). However, some of the cases reported in Europe have been caused by *B. microti* and *B. venatorum* (Herwaldt *et al.*, 2003) . Furthermore, the first cases of human babesiosis were detected in Italy and Austria, and found to be due to *B. venatorum.* This pathogen has been detected in roe deer, which is considered to be a reservoir host for this species (Bonnet, Jouglin, Hostis, & Chauvin, 2007; Herwaldt et al., 2003).

Human babesiosis infection from the rodent parasite *B. microti* has been reported in the USA, with some cases appearing in North-Eastern and Upper Midwest states (Dammin, Spielman, Benach, & Piesman, 1981; Telford et al., 1993). The spectrum of human infection is broad, ranging from an asymptomatic infection to serious inflammation, particularly in people with a weakened immune system due to cancer or HIV-driven AIDS. Indeed, a recent study demonstrated the first isolation of *Babesia divergens* from a HIV patient, where the infection may have existed subclinically for some time (González *et al.*, 2015).

1.2.3.2. *Babesia* life cycle.

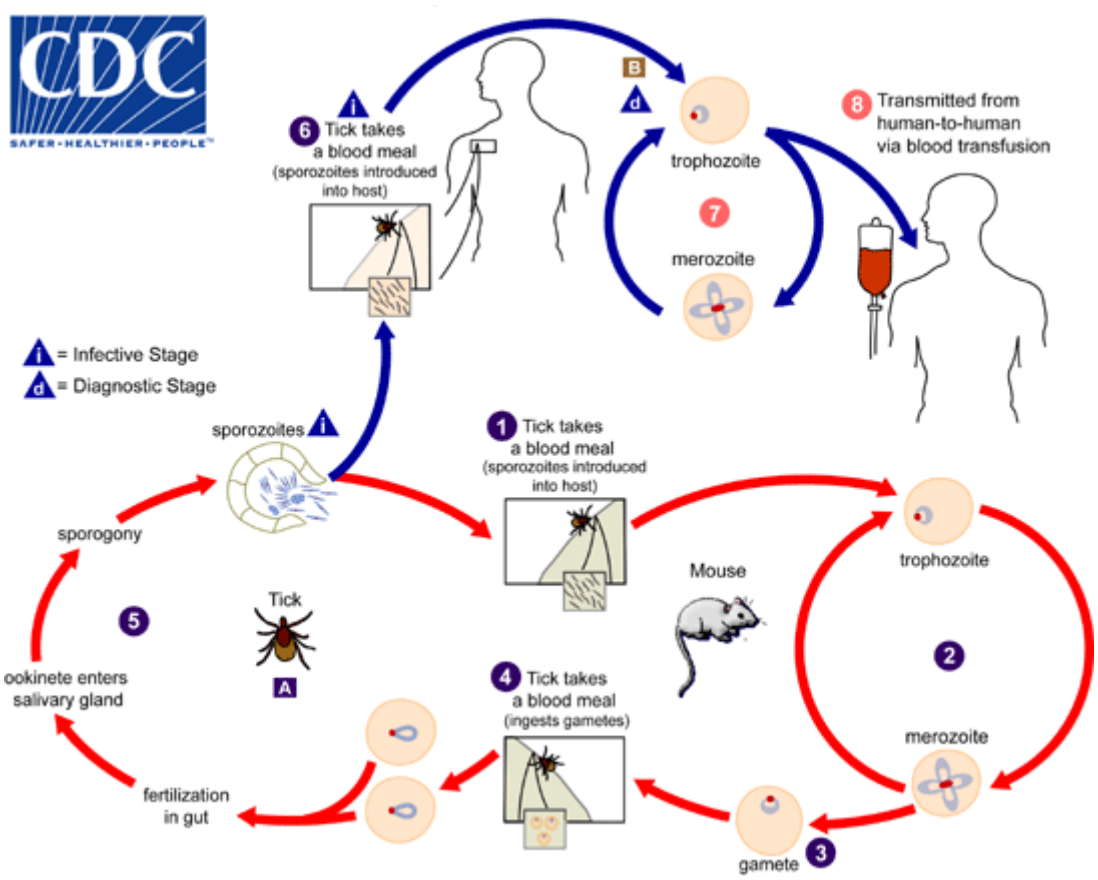


Figure 1.3. The life cycle of *Babesia* species (CDC, 2016).1:The sporozoites enter the host' erythrocytes once the blood meal is taken from the infected host.2. The parasites undergo asexual reproduction inside the red blood cell. 3. In the blood, some of the parasites transforms into male and female gametes.4: The gamete forms enter the tick's body once the blood meal is taken from the infected host, and the fertilization process starts to form the zygote. 5. The zygote will then form the ookinete, which invades the salivary gland and forms the sporozoites.

Two hosts, mammals and ticks, are involved in the life cycle of *Babesia* species (Figure1.3). A large number of studies have been performed in order to understand the life cycle of *Babesia*, however, the information remains incomplete for some *Babesia* species (Bock *et al.*, 2004). Briefly, when an infected tick takes the blood meal, approximately

1000 sporozoites are injected together with the tick's saliva, reaching the bloodstream of the host where they only invade erythrocytes. When the sporozoites are inside the erythrocytes, they begin to differentiate into trophozoites, becoming merozoites after sexual division. A large number of the merozoites continue to replicate in the host by invading new erythrocytes, however, some merozoites stop dividing and become gametocytes that produce gamonts. The second part of the life cycle occurs when the gamonts have then been ingested by a new tick. In the midgut of the tick, the gamonts differentiate into gametes (ray body), which then transform to diploid zygotes. The kinetes are produced from meiosis of the zygote, which can replicate and invade several organs, such as the salivary gland. They are then finally transformed into sporozoites (Berggoetz, 2013).

1.2.3.3. Babesiosis in animals.

Both domestic and wild animals are responsible for the spread of several infectious diseases as they act as important reservoirs for different pathogens such as *Babesia*. *Babesia* species are distributed worldwide, living in free-living mammals, which can cause economic implications due to their impact on morbidity, mortality and weight loss of the animals (Ahmad *et al.*, 2014) . For instance, bovine babesiosis is caused by *B. bigemina* and *B. bovis*, leading to financial losses associated with livestock production in Asia, Africa and South America (Brockelman, 1989). *Babesia bovis* is less common than *B. bigemina* but is considerably more virulent. The main vector of this infection in tropical and sub-tropical regions is the *Boophilus* tick (Ahmad *et al.*, 2014).

Babesia divergens is the most pathogenic species of the *Babesia* genus, known to infect cattle in northern temperate regions (Hunfeld *et al.*, 2008). Recent studies have reported the presence of this parasite in *I. ricinus*, found in forest areas in France, and from wild cervids in Belgium (Bonnet *et al.*, 2014; Lempereur *et al.*, 2012). These studies have suggested that the geographical distribution of the disease is increasing, even in forested areas where cattle are absent. Furthermore, the parasites have been reported to be able to infect sheep, red deer, roe deer, splenectomised rats and gerbils.

Worldwide, 12 species of *Babesia* have been known to cause disease in dogs. Canine babesiosis is usually identified by the morphology of the parasite in the erythrocyte. The most predominant species that causes canine babesiosis in Europe is *B. canis*, which is

considered a large canine piroplasm, whereas *B. gibsoni* is a small piroplasm (Cardoso *et al.*, 2013). *Babesia canis*, *B. microti-like* (*T. annae*) and *B. gibsoni* have been identified in different species of dogs in countries including France, Germany, Austria, Hungary, Spain, Italy, the UK and the USA (Bourdoiseau, 2006; Criado-Fornelio *et al.*, 2003; Hinaidy & Tschepper, 1979; Sánchez-Vizcaíno *et al.*, 2016; Solano-Gallego *et al.*, 2008).

Recent studies have recognised *B. microti-like* species (*T. annae*) as a piroplasm that can cause piroplasmosis. There are multiple names for *B. microti*-like species (*T. annae*), which has been reported as *B. microti*- like (Camacho et al., 2001; Zahler et al., 2000), Spanish dog isolate (Yeagley *et al.*, 2009), *B. annae* (Camacho, 2005), *Babesia* cf. *microti* (Karbowiak *et al.*, 2010) and *T. annae* (Clancey *et al.*, 2010). Furthermore . A recent study used phylogenetic analyses to indicate that these are all the same species and should be reclassified as *Babesia vulpes* sp. nov (Baneth *et al.*, 2015). Consequently, this study will use that particular classification to identify this organism henceforth. The genetic analysis of small canine babesiosis has demonstrated that the *B. gibsoni* and *B. microti*-like (*T. annae)* species are phylogenetically similar to rodent piroplasm *B. microti* (Criado-Fornelio *et al.*, 2003). The first case of *Babesia vulpes* infection was reported in a dog from Germany, which travelled to the Pyrenees in Spain/France. Furthermore, using molecular techniques, *B. vulpes* infection has been reported in North-West Spain (Tabar *et al.*, 2009) and in other countries such as Sweden (Falkenö *et al.*, 2013) ,the USA (Yeagley *et al.*, 2009) , Croatia (Beck *et al.*, 2009) and Portugal (Simões *et al.*, 2011).

Canine babesiosis is transmitted naturally by different tick species, such as *Rhipicephalus sanguineous* which transmits *B. canis*. *Dermacentor* ticks are another vector responsible for the transmission of *B. gibsoni* to different dog species (Homer *et al.*, 2000). Furthermore, different species of ticks, such as *I. hexagonus*, *I. canisuga* and *I. ricinus*, have been suggested to be competent vectors of *B. microti-like* (*T. annae*) species in Spain, and Germany (Birkenheuer *et al.*, 2010; Camacho *et al.*, 2003; Najm *et al.*, 2014).

With regard to the infection of foxes by *Babesia*, several studies have reported cases of *B. vulpes* infection of the red fox (*Vulpes vulpes*) in Spain, which was later also reported in Portugal, Canada, Croatia, Italy, USA, Austria and Germany (Birkenheuer *et al.*, 2010; Clancey *et al.*, 2010; Criado-Fornelio *et al.*, 2003; Dežđek *et al.*, 2010; Duscher *et al.*, 2014; Najm *et al.*, 2014; Tampieri *et al.*, 2008). The prevalence of infection in the red fox population has been reported in Germany, Portugal, North America and Austria as being

46%, 69%, 39% and 50%, respectively (Birkenheuer *et al.*, 2010; Cardoso *et al.*, 2013; Duscher *et al.*, 2014; Najm *et al.*, 2014). Furthermore, *B. vulpes* has been reported to cause infection in grey foxes in the USA (Birkenheuer *et al.*, 2010) . In addition, the first report of *B. canis* in red foxes worldwide was in Portugal (Cardoso *et al.*, 2013). Detection of this pathogen in the fox population suggests that a sylvatic cycle of this parasite exists in the fox population, and that foxes act as a host reservoir (Cardoso *et al.*, 2013).

1.2.3.4. Symptoms of *Babesia* infection.

The symptoms of *Babesia* infection can range from a subclinical infection to fulminating disease, which can result in death (Vannier *et al.*, 2008). Most infected people become ill for between 1 and 4 weeks after being bitten by an infected tick, although delayed presentation is possible (González *et al.*, 2015).Transmission of *Babesia* species by blood transfusion from an infected donor is considered to be the second route of infection. The symptoms can appear between 1 and 9 weeks following a tick bite (Herwaldt *et al.*, 2011). Occasional symptoms of the disease include abdominal pain, depression, chills, sore throat and weight loss (Ruebush *et al.*, 1977).

1.2.4. *Bartonella*.

The *Bartonella* genus, belonging to the Alphaproteobacteria class (Proteobacteria phylum) includes more than 30 species of Gram-negative intracellular bacteria (Sofer *et al.*, 2015), Pathogens of this genus normally parasitize different cell types in the host, including the endothelial cells, macrophages and erythrocytes (Saisongkorh *et al.*, 2011). They use these cells to avoid and hide from the host immune system, allowing them to establish a chronic infection. Among the 30 species of *Bartonella* currently known, 15 species are known to be pathogenic to humans (Table 1.4).

Table 1. 4. Species of the *Bartonella* genus confirmed as human pathogens.

***Bartonella* species**	**Vector**	**Host**	**Reference**
B. tamiae	Tick and mite	Rodent	(Colton *et al.*, 2010)
B. quintana	Cat flea, body louse, tick	Human	(Ohl & Spach, 2000)
B. alsatica	Pulex flea	Rabbit	(Breitschwerdt *et al.*, 2007)
B. rochalimae	Pulex flea	Dog and human	(Henn *et al.*, 2009)
B. henselae	Cat flea, tick	Cat	(Mexas *et al.*, 2002)
B. elizabethae	Rodent flea	Rodent	(Kosoy, Iverson, *et al.*, 2010)
B. tribocorum	*Xenopsylla cheopis*	Rat *(Rattus norvegicus*)	(Kosoy et al., 2010)
B. vinsonii arupensis	flea/tick	White-footed mouse	(Boulouis *et al.*, 2005; Chomel *et al.*, 2004)
B. koehlerae	Cat fleas	Cat	(Rolain *et al.*, 2003)
B. washoensis	flea	Ground squirrel	(Kosoy *et al.*, 2003)
B. vinsonii berkhoffii	tick	Dog and coyote	(Malania *et al.*, 2016; Welch *et al.*, 1999)
B. bacilliformis	Sand fly	Human	(Chamberlin *et al.*, 2002)
B. grahamii	Rodent flea	Rodent	(Kosoy et al., 2010)
B. rattimassiliensis	Sand fly	Human	(Boulouis *et al.*, 2005)
B. clarridgeiae	Cat flea	Cat	(Boulouis *et al.*, 2005)

Several haematophagous arthropods have been implicated in the transmission of different *Bartonella* species, including fleas, ticks, lice and sand flies (Billeter *et al.*, 2008, , 2012; Morse *et al.*, 2012). The bacteria are maintained in natural environments by infecting a wide variety of reservoirs, such as rodents, canines and felines (Chomel *et al.*, 2014). *Bartonella* species have been associated with important epidemic diseases, such as Oraya fever, caused by *B. bacilliformis* and are primarily found in the Andes Mountains, while *B. quintana* has been identified as the causative agent of trench fever (Karem *et al.*, 2000). Furthermore, some studies have provided evidence of vector specificity for example, *B. quintana, B. bacilliform* and *B. henselae* are associated with *Pediculus humanus* (body louse), *Lutzomia verrucarum* (sand fly) and cephalides felis (cat flea), respectively (Alexander, 1995; Chomel *et al.*, 1996; Maurin & Raoult, 1996).

1.2.4.1. Animal Bartonellosis.

The prevalence of *Bartonella* species in rodents in the UK and Southeast USA has been reported to be 62.2% and 42.2%, respectively (Birtles *et al.*, 1994; Kosoy *et al.*, 1997). In addition, *Bartonella* species have been reported in wild animals in the USA, including coyotes, lions, black-tailed deer and bobcats (Chang *et al.*, 1999; Gurfield *et al.*, 1997), and the prevalence in the cat population in Hawaii, France, San Francisco (USA) and Indonesia has been reported to be 42.3% (Demers *et al.*, 1995) , 53% (Heller *et al.*, 1998), 85.7% (Koehler *et al.*, 1994) and 64% (Marston *et al.*, 1999), respectively. Several species of *Bartonella* have been detected in wildlife and domestic animals (Table 1.4). Furthermore, Mexas et al (2002) suggested that *B. elizabethae* should be added to the list of Bartonella species, including *B. clarrdgeiae, B. henselae* and *B. vinsonii*, which normally infect dogs. Molecular studies were performed by Henn et al (2009) for characterising *Bartonella* infection in raccoons, coyotes and red foxes from the USA and Paris (France), and showed that 2/21 of coyotes, 11/42 of raccoons, and 1/1 red fox were infected by *B. rochalimae*.

In a study performed in the Middle East, amplification of the ITS region by PCR showed the presence of different *Bartonella* species, such as *B. rochalimae, B. henselae* and *B. elizabethae,* in a variety of flea species that normally infest dogs (Sofer *et al.*, 2015).

The infection of different rodent species from different parts of the world with *Bartonella* has been extensively studied, which has illustrated the high prevalence of *Bartonella* species among rodents (Bai *et al.*, 2011; Castle *et al.*, 2004; Ying *et al.*, 2002). Five rodent-associated species of *Bartonella* are considered to be causative agents of human infection, including *B. grahamii, B. washoensis, B. tribocorum. B. vinsonii* subsp. arupensis and *B. elizabethae* (Daly *et al.*, 1993; Kosoy *et al.*, 2003; Welch *et al.*, 1999). These species have been associated with a wide range of symptoms in infected humans, including neurological symptoms, fatigue, endocarditis and muscle and joint pain (Anderson & Neuman, 1997; Breitschwerdt *et al.*, 2007).

In Asia, rodent population have been reported to be infected by a diverse range of *Bartonella* species in several countries such as Thailand, China, Bangladesh, Japan and Israel (Jiyipong *et al.*, 2014). Some studies have investigated the prevalence of the infection in rodent populations in some countries, with overall prevalence ranging from 6% to 47% in Korea and China (Chae *et al.*, 2008; Ying *et al.*, 2002). Rodents from China, Taiwan, Korea, Russia and Japan had a high prevalence of infection ranging from 8.6% to 82.3% (Jiyipong *et al.*, 2014) . Different species of rodents such as *Myodes* spp, *Eothenomys* spp and *Apodemus* spp have been reported as reservoirs for *Bartonella* species (Jiyipong *et al.*, 2014). Investigation of *Bartonella* spp infection in the West and Southern parts of Asia is very limited (Table 1.5). However, zoonotic bacteria have been detected in some of these regions. For example, the presence of *B. elizabethae* was confirmed by PCR in 16% (10/68) and 25% (1/4) of the rat (*R. rattus*) and *Apoemus cahirinus*, while the prevalence of *B. tribocorum* was recorded as 24% (19/79) (Harrus *et al.*, 2009; Morick *et al.*, 2009).

The first investigation of *Bartonella* infection in rodent species from tropical areas was performed in Yunan (South-Western China) by Ying et al (2002). This study reported that *Rattus rats* were infected with *B. elizabethae*, which can infect human populations. Consequently, many studies were conducted in different tropical areas including Bangladesh, Indonesia and Thailand, to detect the pathogen in rodent species (Bai *et al.*, 2007, , 2009; Castle *et al.*, 2004; Winoto *et al.*, 2005). The first report that investigated the presence and diversity of *Bartonella* infection in six species of rodents from Thailand was performed by Castle et al (2004). This group demonstrated that 17 out of 195 (8.7%) of different rodent species, specifically 3/24 *R. rattus* (12.5%), 12/147 (8%) *B. indica* and

2/11 *R. losea* (18.2%), were infected by several *Bartonella* species such as *B. grihami* and *B. elizabethae*, which were known to cause human illness.

Sequence analysis of the ssrA gene of *Bartonella* isolates demonstrated that rodents in Thailand harboured different strains of *Bartonella*, including *B. rattimassiliensis*, *B. queenslandensis*, *B. phoceensis*, *B. tribocorum* and a new *Bartonella* species (GU056190). In this study, a total of 619 rodents were collected from four regions, with the most prevalent species of rat reported to be 279 *Bandicota*, 163 *R. rattus* and 96 *R. exulans*, for which around 17% (109/619) were infected with *Bartonella*. The highest disease prevalence was detected in 53/163 (32.5%) *R. rattus* and 5/149 (35.7%) *B. savilei*, with a high prevalence of infection also reported in 42/279 (15.1%). *Bartonella indica* was reported in 5/40 *R. norvegicus* animals (Klangthong *et al.*, 2015).

In Bangladesh, the prevalence of *Bartonella* infection was reported to be 42.8% in 201 mammalian species (99 black rats, 12 house mice, 76 lesser bandicoot rats and 14 house shrews). The highest prevalence of the infection among these species was 63.2% in bandicoot rats, 32.3% in *R. rattus* and 42.9% in house mice (Bai *et al.*, 2007). Moreover, prevalence of the *B. elizabethae* was reported in 48 out of 76 (63.2%) *Bandicota bengalensis* animals in Bangladesh (Winoto *et al.*, 2005).

Table 1.5.The prevalence and diversity of *Bartonella* species in some Asian countries.

Country	**Host**	***Bartonella* species**	**Prevalence**	**Reference**
Israel	*Rattus rattus*	*B. elizabethae*	16%	(Harrus *et al.*, 2009)
	Apodemus cahirinus	*B. elizabethae*	25%	(Morick *et al.*, 2009)
	R. rattus	*B. tribocorum*	24%	(Morick *et al.*, 2009)
Bangladesh	*R. rattus,*	*B. tribocorum*	32.3%	Winoto et al., 2005)
	Bandicota bengalensis	*B. elizabethae*	63.2%	Winoto et al., 2005)(Chae *et al.*, 2008)
Indonesia	*R. tanezumi*	*B. phoceensis*	10.3%	(Winoto *et al.*, 2005)
	R. norvegicus	*B. rattimassiliensis*	2%	(Winoto *et al.*, 2005)
Thailand	*B. indica*	*B. elizabethae*	8.1%	(Castle *et al.*, 2004)
	B. savilei	*B. elizabethae*	57.1%	(Bai *et al.*, 2009)
	R. norvegicus	*B. tribocorum*	86.4%	(Bai *et al.*, 2009)
	R. tanezumi	*B. grahamii*	12.5%	(Castle *et al.*, 2004)
	R. tanezumi	*B. coopersplainsensis*	8.2%	(Saisongkorh *et al.*, 2009)
Cambodia	*Berylmys berdmorei*	*B. elizabethae*	9.1%	(Jiyipong *et al.*, 2012),
	R.argentiventer	*B. tribocorum*	9.5%	(Jiyipong *et al.*, 2012)
	B. savilei	*B. coopersplainsensis*	9.5%	(Jiyipong *et al.*, 2012)

In Georgia (USA), Malania et al (2016) found that 28 out of 68 rodents were infected with *Bartonella* species, providing strong evidence for the presence of different *Bartonella* strains including *B. grahamii, B. elizabethae* and *B. tribocorum*, in addition to an unknown strain that was currently circulating in this environment.

Amplification of the citrate synthase (gltA) gene by PCR illustrated the presence of *Bartonella* species in 63/325 *R. norvegicus* and 11/92 *R. rattus* animals from Portugal and the USA, with the *Bartonella* isolated from *R. norvegicus* shown to be similar to *B. elizabethae* (Ellis *et al.*, 1999).

Two sites in Kenya (Asembo and Kibera) were investigated for the presence of *Bartonella* infection. In Asembo, 10 out of 49 trapped rodents were positive for different *Bartonella* species, including *B. elizabethae, B. tribocorum* and *B. birtrlesii-like*, whereas the most common species of *Bartonella* detected in Kibera were *B. tribocorum, B. queenslandensis* and *B. elizabethae* (Halliday *et al.*, 2015).

1.2.4.2. Host specificity.

Host specificity for *Bartonella* infection in rodent communities has been investigated in different parts of the world. A study by Birtles in 1994 showed that three *Bartonella* species, including *B. doshiae*, *B. grahamii*, and *B. taylorii* were found in all dominant species of rodent species such as *Microtus agrestis, Apodemus sylavaticus, Neomys fodiens, Myomys glareolus* and *Apodemus flavicotlis*. Furthermore, similar results were reported in rodent species from central Sweden, showing that *Microtus glareolus, Mus musculus* (house mice), *Apodemus sylvaticus* and *Apodemus flavicollis* were frequently infected with *B.grahamii* (Holmberg *et al.*, 2003).

Studies of *Bartonella* infection in rodent populations from North America have shown a different scenario of *Bartonella* species in relation to host specificity, showing that bartonella species were detected in specific rodent genus such as Peromyscus (mice) and Neotoma (rats) (Bai *et al.*, 2008; Kosoy *et al.*, 1997). These studies demonstrate important evidence for host-specific relationships that exist between those bartonella species and their rodent reservoir.

In Vietnam, the relationship between *Bartonella* infection and rodents appears to similar from what has been shown in South-Western China where B*artonella* species were

detected in *Rattus* rats and classified as *B. elizabethae*, thus illustrating a very specific relationship (Ying *et al.*, 2002).

These studies illustrate the potential role of different rodent species as a reservoir host for *Bartonella* species, which can be pathogenic to humans. In addition to this, due to the close relationship between rodents and humans around the world, further studies related to rodents infected with *Bartonella* are important in order to determine whether rodents that might serve as the main source of zoonotic infection in humans (Malania *et al.*, 2016).

1.2.5. Candidatus midichloria

Midichloria is considered to be an intracellular bacterium symbiont within the order Ricketsiales (Beninati *et al.*, 2009). The bacteria was first reported in *I. ricinus* in 1979 (Lewis, 1979). Furthermore, the bacteria have been observed in other haematophgous arthropods such as the tabaind fly (Hornok *et al.*, 2008), mites, (Reeves *et al.*, 2006) and bugs (Richard *et al.*, 2009).The bacteria is found in the reproductive tissue of the female tick and it can invade and destroy mitochondria in ovarian cells (Sacchi *et al.*, 2004). It has been reported in 100% of tested *I.ricinus* females from different geographical distribution and it was reported in 44% of male ticks ((Lo *et al.*, 2006). A molecular study by Cafiso (2016) was able to detect the bacterium in 7 out of 92 screened ticks, including the first record of this bacteria in the soft tick *Ornithodoros maritimus* (Cafiso *et al.*, 2016).

Laboratory studies of the bacteria in ovaries of infected ticks using electronic microscopy demonstrated different characteristic of the bacterium, which can degrade several mitochondria in this organism (Beninati *et al.*, 2004). Midichloria is the only bacterium that can invade the mitochondria of any multicellular host (Beninati *et al.*, 2009).

Recent serological and molecular studies have illustrated that midchloria DNA was observed in a mammalian host (Mariconti *et al.*, 2012). However, its role here is still unknown. A serological test in Germany has shown that 48 out of 168 (28%) human serum samples were positive for this organism (Serra *et al.*, 2016) . Human samples have been investigated for the presence of midicholoria DNA using an ELISA assay, where the sera were positive in 82 out of 274 tested samples, which were collected from Germany (Serra & Bazzocchi, 2017).

There has been some debate as to whether midichloria (also known as *M. mitochondrii*) could be potential pathogens of man (Mariconti *et al.*, 2012). *M. mitochondrii* was present in the salivary glands of *I. ricinus* and seropositivity against *M. mitochondrii* was highly prevalent in humans colonised by *I. ricinus* (58%), while it was very low in healthy individuals (1·2%). This organism should be regarded as a group of antigens inoculated into the human host during the tick bite.

1.3. Vector transmission.

1.3.1. Ticks.

Tick species were the first arthropod species to be described as a vector of infectious disease. Currently, in addition to mosquitoes, they are considered to be the main arthropod vectors for transmitting pathogens to humans and domestic animals worldwide (Colwell *et al.*, 2011; Jongejan & Uilenberg, 2004). Ticks are obligate blood-feeding arthropods, and are of considerable medical and veterinary importance due to their ability to transmit a wide variety of pathogens of public health importance, such as bacteria, viruses, protozoa, rickettsias and spirochaetes (Bonnet et al., 2007; Johnson, Schmid, Hyde, Steigerwalt, & Brenner, 1984; Labuda & Nuttall, 2004; Mehlhorn & Armstrong, 2010). Ticks belong to the order Parasitiformes and sub-order Ixodiade, which are divided into three families; hard ticks, soft ticks, and Nuttalliellidae (Mehlhorn & Armstrong, 2010).

1.3.1.1. Hard Ticks.

Hard ticks (*Ixodidae*) comprise approximately 683 species belonging to 12 genera, such as *Ixodes, Hyalomma,* and *Dermacentor* (Horak *et al.*, 2002). All hard tick species belong to the Ixodidae family. The capitulum of ticks in this family is dorsal and can be recognised from a top-down perspective. They also have a scutum, which covers the entire dorsal body in the male gender, while only the leg-bearing section is covered in females. In females, the cuticle of the abdomen contains extensive folds, allowing the abdomen to extend when they feed on the host (Bush, 2001). During the life span of hard ticks, they can attach and feed on several hosts (usually between 1-3 days), belonging to either the same or a different species. Hard ticks can feed for a long time, and the blood meal takes

between 4–7 days to ingest. The specificity of the host is very low for most tick species, and ticks can be found on any available mammalian host e.g. humans, deer, and sheep. The larvae and nymph stages of the hard tick are found on small mammal species, whereas adult ticks are associated with larger mammals (Bush, 2001).

Most species of hard ticks have three hosts, with the exception of species in the *Boophilus* genus, which only have one host. For *Boophilus* spp., all developmental stages of the life cycle occur in the same host. The life cycle of *Ixodes* ticks involves four stages (egg, larva, nymph, and adult). Three different hosts are required by *Ixodes* species in order to complete their life cycle (three-host life cycle). Briefly, the first stage begins when the female ticks take the blood meal, with the pathogen leaving the host in order to lay their eggs. The eggs are laid in the ground, and later hatch to release the larvae. Characteristically, larvae at this stage only have six legs, whereas they have eight legs at later stages. The larvae seek a small mammal or bird to feed on for a couple of days until they become engorged, after which they detach from their host and moult into their second stage (nymph) on the ground. The nymph searches again for a suitable host to feed, then moults to the adult stage, which is the final stage of the life cycle. The size of the host differs throughout the life cycle. For example, ticks in the nymph stage look for larger hosts than those at the larval stage, and they often feed from 3 to 4 days, whereas the adult ticks attach to a larger mammal host. Epidemiological studies of the prevalence and distribution of *Ixodes* species have illustrated several differences between species of the *Ixodes* genus. For instance, *I. ricinus* searches for hosts on vegetation, whereas other species, such as *Ixodes hexagonus* and *Ixodes canisuga*, are considered to be nidicolous, remaining in the burrow or nest of their host (Gern *et al.*, 1996).

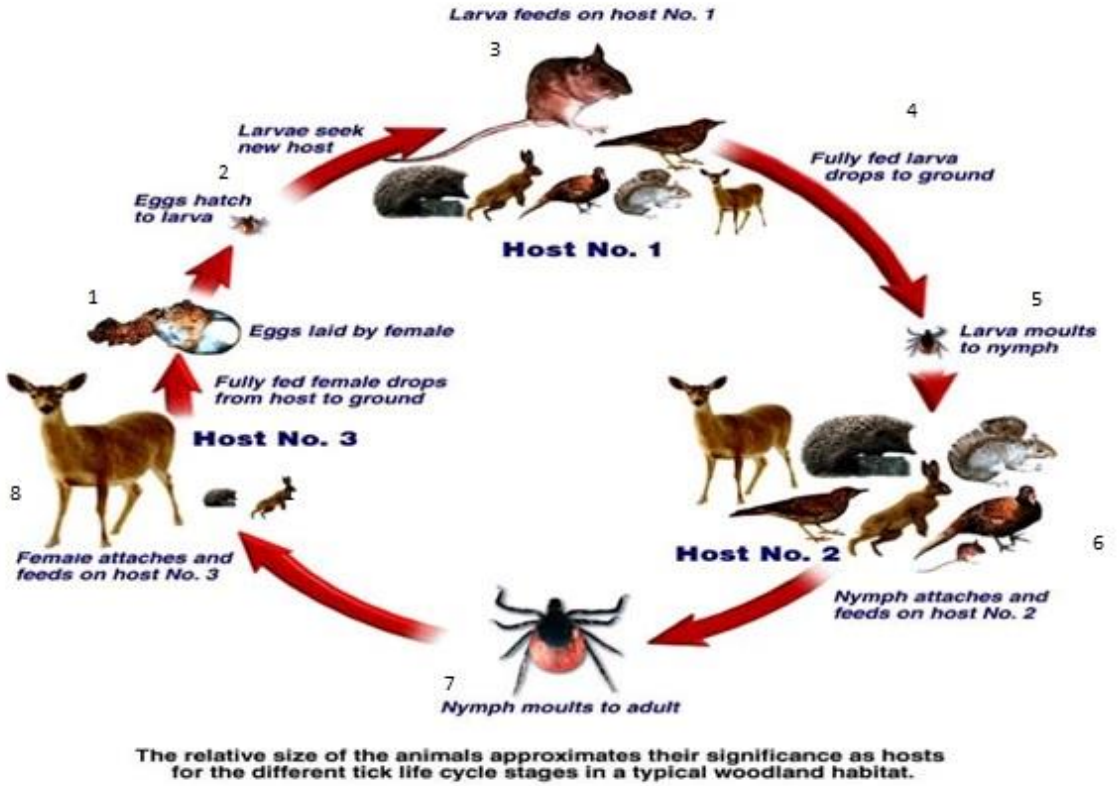


Figure 1.4. The life cycle of Ixodes ticks (Gray 1991). Female ticks lay the eggs in the ground, the eggs hatch (1), and the larva stage appears (2). The larva search for suitable hosts (3) and take blood. The tick then drops (4) and moults to second stage (nymph (5)) which searches for another host (6) looking for blood. This then drops from the host after the bloodmeal and moults into an adult tick (7), which infects a new host looking to mate.

Some tick species complete their life cycle on only two individual hosts (two-host life cycle), in which both larvae and nymphs feed on the same host, but the adults feed on a second host. This type of life cycle has been recognised in several tick species including *Rhipicephalus evertsi evertsi* and *Hyalomma marginatum rufipes* (Walker, 2003).

The third type of tick life cycle is known as the "one-host life cycle", where at all stages of their life cycle, ticks feed and moult on the same host. Three examples of this type are *Rhipicephalus (Boophilus) decoloratus, R. (B.) microplus* and *Margaropus winthemi* (Berggoetz, 2013).

The genus *Ixodes* can be found in many different parts of the world. Although many species of *Ixodes* cannot cause serious damage to their host, several species are considered vectors of the spirochaete *Borrelia spp.*, the causative agent of Lyme disease. For example, in North-East America, *Borrelia burgdorferi* is transmitted by *I. scapularis* (black-legged tick), while *I. pacificus* is responsible for transmitting the pathogen in North-West America (Table 1.6). In Europe and Japan, *I. ricinus* and *I. persulcatus*, respectively, are the competent vectors for Lyme disease (Bush, 2001).Furthermore, different species of *Dermacenter*, such as *D. andersoni*, *D. variabilis* and *D. occidentalis*, are competent vectors for Rocky Mountain spotted fever, in addition to being a vector for numerous viral and bacterial infections (Bush, 2001).

Table 1.6. Hard ticks and the infection they transmit .

Hard tick (*Ixodidae*)	**Transmitted infection**	**Reference**
Dermacentor andersoni	*Anaplasma marginaleb, Francisella tularensis, Rickettsia rickettsii,*	(Jongejan & Uilenberg, 2004; Petersen *et al.*, 2009)
Dermacentor nuttalli	*Rickettsia sibirica*	(Jongejan & Uilenberg, 2004)
Dermacentor silvarum	*Rickettsia heilongjiangensis* and *R. sibirica*	(Parola *et al.*, 2005)
Amblyomma americanum	*Borrelia lonestari, Ehrlichia chaffeensis, F.* *tularensis, Rickettsia parkeri* and *R. rickettsii*	(Estrada-Peña & Jongejan, 1999; Jongejan & Uilenberg, 2004)
Amblyomma triste	*R. parkeri*	(Labruna, 2009)
Haemaphysalis concinna	*Anaplasma phagocytophilum, F. tularensis,* and *R. sibirica*	(Barandika *et al.*, 2008; Gyuranecz *et al.*, 2011; Jongejan & Uilenberg, 2004)
Hyalomma anatolicum	*Theileria annulata,* and *Theileria lestoquardi*	(Jongejan & Uilenberg, 2004; Labuda & Nuttall, 2004)
Hyalomma marginatum	*Rickettsia aeschlimannii, T. annulata,*	(Jongejan & Uilenberg, 2004)
Ixodes ricinus	*A. phagocytophilum, Babesia divergens, B. microti,* *B. burgdorferi sensu lato (s.l.), F. tularensis,* and *R. helvetica,*	(Jongejan & Uilenberg, 2004; Milutinović *et al.*, 2008)
Ixodes hexagonus	*Borrelia burgdorferi sensu lato*	(Jongejan & Uilenberg, 2004)
Ixodes scapularis	*A. phagocytophilum, B. microti, B. burgdorferi s.l.*	(Jongejan & Uilenberg, 2004)
Rhipicephalus microplus	*A. marginale, B. bigemina, B. bovis* and *Theileria equi*	(Jongejan & Uilenberg, 2004)
Rhipicephalus turanicus	*R. conorii* and *R. massiliae*	(Estrada-Peña & Jongejan, 1999)

1.3.1.2. The importance of the tick population on vertebrate health.

The feeding behaviour of several tick species makes them an important health issue for both humans and domestic animals. When the blood meal is taken, ticks can cause disease to the host by damaging the skin, causing local pain and irritation. More serious diseases occur when microorganisms are introduced into the body by an infected tick, which can lead to systemic and fatal diseases in the host.

All feeding stages of different tick species are considered parasitic, as they feed only on the blood of their host to complete their life cycle. They firstly attach to the skin and begin to cut the skin with their mouthparts, after which an adhesive secreted in the saliva is used to assist attachment of the tick to the skin, while a powerful anaesthetic often means the host is unaware that the tick is attached. The whole process is termed salivary-assisted transmission (Kazimírová & Štibrániová, 2013). The sharp chelicerae at the end of the mouthpart make a hole in the dermis, which breaks blood vessels close to the skin surface. This allows the attached tick to feed on the released blood.

A variety of infectious agents, such as viruses, protozoa, spirochaetes and rickettsiae, are extensively transmitted by tick species compared with any other group of haematophagous arthropods. Severe toxic conditions, such as allergy, paralysis and irritation, have also been associated with tick-borne diseases (Jongejan & Uilenberg, 2004).

The importance of tick-borne diseases for humans, wildlife and domestic animals is measured by the rate of morbidity and mortality. The spectrum of tick-borne disease in these populations has increased recently due to zoonotic tick-borne diseases, including Lyme disease, borreliosis, babesiosis, anaplasmosis and ehrlichiosis, which have recently gained increasing attention by veterinarians and physicians (Torres, 2012). The development of new molecular techniques has facilitated the detection of new species and strains of disease-causing organisms around the world (Duh *et al.*, 2010; Pacheco *et al.*, 2011). In North-East America, *Ixodes scapularis* (blacklegged tick) is a competent vector for several pathogens, such as *B. microti*, *Anaplasma phagocytophilum* and *Borrelia burgdorferi*, all of which can infect humans (Piesman & Eisen, 2008). Furthermore, the presence of *B. burgdorferi*, considered to be the causative agent of Lyme disease, has been reported in the northern hemisphere including countries such as Scotland. Indeed, the incidence of Lyme disease in Scotland has been increasing annually for several years

(Slack *et al.*, 2011). This pathogen has now been classified as a member of the *B. burgdorferi sensu lato* species complex along with human pathogens including *B. garinii*, *B. afzelii* and *B. burgdorferi* (Bergström *et al.*, 2002; Wang *et al.*, 1999).

1.4. Investigated host.

In the study different host species have been investigated include rodents (bank vole and wood mice), red foxes, Libyan jird, desert hedgehogs and *I.ricinus* ticks were investigated for the presences of haemoparasitic agents.

1.4.1. Bank vole (*Myodes glareolus*).

Bank voles can be found from Central Asia to Europe, including the GB. They have also been reported in areas such as the remote islands of Bute, Skomer, Wight and Raasay (Sibbald *et al.*, 2006). The population of this species exceeds 23 million individuals in the UK, although their density varies across the islands, with 3.5 million bank voles estimated to live in Scotland, 1.75 million in Wales and 17.75 million in England (Sibbald *et al.*, 2006). The large English population is believed to be associated with the presence of large extensions of hedgerows in arable landscapes (Bellamy *et al.*, 2000; Flowerdew & Gardner, 1978). This species prefers to inhabit mature, mixed-deciduous woodlands, associated with thick shrubs or fields (Sibbald *et al.*, 2006).

Adult bank voles are 10–11 cm long and weigh 17–20 g, with males and females presenting similar body sizes. Their body is covered by a brown or grey coat, and they are characterised by their prominent ears and short tail (3–4 cm long), which is shorter than their full body length (MacDonald, 2009). Bank voles can feed on a wide range of foods, including nuts, seeds and roots. During winter, they forage by burrowing underground, and their dietary habits vary depending on the season and their location (MacDonald, 2009). This species of rodent has a short lifespan, ranging from only a few months to up to 2 years (Yanagihara & Masuzawa, 1997).

Bank voles are polygamous (MacDonald, 2009). Females are known to defend their territory, which often overlaps with that of another female. Males, on the other hand, defend territories that often overlap with the territories of different females (Horne & Ylönen, 1996). The breeding season is between late April and September (Oksanen *et al.*, 2001). The gestation period can range between 17 days, with optimal nutrition, and 24

days if the female becomes pregnant while lactating or during postpartum oestrus (MacDonald, 2009). The litter size can of bank voles vary between 1–10 pups, averaging 4–8 pups (Oksanen *et al.*, 2001).

1.4.2. Wood mice (*Apodemus sylvaticus*).

The wood mouse, or long-tailed field mouse, is a common species that is widespread throughout the UK. The estimated population size is approximately 38 million individuals. In England only, the population of this species is estimated at 15 million individuals, whereas in Scotland and Wales the population is estimated to be 15 million and 3.5 million individuals, respectively (Harris, 1995).

Wood mice can adapt to, and inhabit, most environments, including heathers, arable land, rocky mountains, woodlands and blanket bogs (Harris, 1995). Previous studies have shown that this species can be found in both wet and dry regions, including deciduous woodlands (Sibbald *et al.*, 2006). An urban ecological study showed that wood mice can be found in conurbations in habitats such as scrubs, churches and woodlands (Baker *et al.*, 2003).

The body length of the wood mouse is between 4.5–8.2 cm, and their tail length can range from 2.8–6.5 cm. Their abdominal fur is light grey or white, and often presents yellow tinges on the throat. They have characteristically large eyes and ears, which confer excellent night vision and assist in predator avoidance (MacDonald, 2009).

Wood mice usually reproduce between March and October, after multiple mating occurrences. Annually, females can produce up to four litters, and the gestation period lasts between 21–26 days. The litters are born with a thin coat of dark fur and closed eyes, which open after 13 days (Nowak, 1999). The lifespan of wood mice is approximately 1 year.

Wood mice are omnivores that feed on seeds, nuts, grains, roots, fruits and insects. This species provides an important ecosystem service by transporting and burying tree seeds (Khammes & Aulagnier, 2007). However, they have been traditionally viewed as a pest, as they cause serious damage to orchards and farmlands, decimating the crops (Nowak, 1999).

1.4.3. Libyan jird (*Meriones libycus*)

The Libyan jird belongs to the subfamily *Gerbiilinae*, which comprised 95 species classified in 14 genera. They have been found in desert and savannas areas of Africa, and they have been reported in Asian deserts and steppes (Macdonald, 2006).

The Libyan jird has a similar body size (from 6.2-7.5cm to 15-20cm) to the gerbil species such as Mongolian gerbil. They have been reported to have large eyes, fine fur, elongated hind legs and a broad head (Firouz, 2005; Macdonald, 2006). The upper part of the body is brown while the underneath of the animal is greyish/white in colour, and the end of the tail normally ends in a black tuft (Firouz, 2005; Macdonald, 2006). The tail length is between 7.2-9.5 cm to 16-22 cm, and males and females have similar weights that range from 8-11g to 115-19g (Macdonald, 2006). They can adapt to desert habitats that normally have high temperatures and less available water sources. However, they have special body characteristics to deal with these kinds of habitats throughout the extraction of the water from their food, and they normally do not sweat, which lets them adapt to minimization of water loss in this environment (Macdonald, 2006). Their dietary composition involves roots, fruits, seeds and occasional insects (Macdonald, 2006).

Little information is available regarding the breeding behaviour of this species, although other jirds have been reported to breed year round in some regions or can breed during winter and spring months (Macdonald, 2006). Two to three litters a year are produced by the females after a gestation period of 31days (Nowak, 1999). At birth, the young are born blind and naked before their eyes open after two weeks (Macdonald, 2006).

Harrison (1972) reported 15 species of rodent in Saudi Arabia, with the most distributed species being *Rattus rattus* followed by *Mus musculus* and *Rattus norvegicus B.*

Furthermore, five rodents were captured south west part of Saudi Arabia. The most dominant species identified were *Gerbillus dasyurus* and *Acomys dimidiatus homericus* and the least prevalent were *Meriones rex buryi , Praomys fumatus Yemeni* and *Eliomys melanurus* (Al-Khalili *et al.*, 1984).

In a recent survey conducted by (Alahmed & Al-Dawood, 2001) in 2001, six species of rodent were collected from different areas in the Wadi Hanifah (Valley) region of Riyadh. These included *Rattus rattus, Mus musculus, Acomys dimidiatus, R.rattus alexandrinus, R.rattus frugivorous* and *Meriones libycus*. It was also reported that some ectoparasites

such as flea species (*Xenopsyllus*) were detected on *R. rattus alexandrinus* while *R. rattus frugivorous* and *Acomys dimidiatus* were infected with species of ticks (*Rhipicephalus turanicus*).

In another study performed by Al-Rajhi et al (1993) , four rodent species were collected. These were *Meriones libycus, Mus musculus, R .rattus* and *Acomys dimidiatus*. This compared with other collections by El-Bahrawy & Al-Dakhil (1993) who were able to capture seven rodent species including *Jacullus jacullus, Gerbillus spp, Acomys dimidiatus. R. norvegicus, Merioness spp., Mus musculus and R. rattus.* However, it is clear that there has not been any published evidence of a more recent collection of such rodents in Saudi Arabia.

1.4.4. Desert hedgehogs (*Paraechinus aethiopicus*).

A hedgehog is a member of the mammal species that belongs to the subfamily Erinaceinae, order Eulipotyphta. The desert hedgehog can be found in several parts of Africa including Egypt, Sudan, Morocco, and Eritrea. It has also been reported in the Middle East and in some regions of the Arabian Peninsula (Stone, 1995; Vriends & Heming-Vriends, 2000).

The hedgehog is easily recognized by its elongated snout and spiny coat which can prevent it from being eaten by predators such as foxes, as it can curl into a spiny ball when threatened (Macdonald.2006). The body length of desert hedgehog is between 15-25 cm with tail length range from 1-4 cm. Males can weigh up to 535 g while females can be up to 310 g (Vriends & Heming-Vriends, 2000). The legs are long and dark and they have short and rounded ears (Nowak, 1999). The ventral surface of the body is patterned with black or white colouring (Vriends & Heming-Vriends, 2000).

The desert hedgehog is a nocturnal species. Their diet can include a wide range of invertebrate and vertebrates species such as insects and nesting birds (Nowak, 1999). It also has been demonstrated that between January and February, the desert hedgehog enters a period of hibernation when the weather is cold.

The breeding season starts in March and the females can give birth to up to six young, after a gestation period of about 30-40 days. At birth, the young are born blind and the spines are located under the skin in order to protect the female from any damage during the birthing process. The young desert hedgehog can open it’s eyes after around 21 days and they are weaned after about 40 days (Vriends & Heming-Vriends, 2000).

1.4.5. Red foxes (*Vulpes vulpes*).

Foxes are omnivorous mammals that belong to different genera of the Canidae family. They occur in different parts of the world, including Asia, Europe and some parts of Africa (Larivière & Pasitschniak-Arts, 1996). Farmland, tundra, semi-arid deserts and metropolitan areas are the most common habitats for fox species. Foxes have also been found in habitats including savannas, alpine zones, upland fields, coastal beaches and river banks in England (DeGraaf & Yamasaki, 2001). Food availability and suitable den sites are considered to be the most important factors that affect habitat selection by these species (Whitaker & Hamilton, 1998). They prefer to make dens in forests that are close to open areas or areas which provide thicker cover (Voigt & Broadfoot, 1983). Furthermore, they are increasingly common in more urban areas, where there may be more opportunity to interact with humans (Inger *et al.*, 2016).

The body size of red foxes varies throughout their geographic range, with females typically smaller than males. Their fur is a reddish-brown colour, and can vary from a brown-red colour to a yellow-grey colour. The genus *Vulpes* has three main colour morphs, which are a mixture of different colours (greyish brown with black hairs down the back and across the shoulders), red and silver (Johnson & Hersteinsson, 1993). Red foxes generally have a white chest, long legs and slender black lower legs, with a long, bushy and thick tail. The body length including the adult head ranges from 455–900 cm, with a tail length of 300–500 mm and a body weight of 3–14 kg (Nowak, 1999). The red fox species found in the Middle East is smaller than those in North America and Europe (Macdonald, 2006).

The red fox can feed on a variety of plants and prey, including rodents, insects, frogs, fish, birds, worms, fruit and seeds (DeGraaf & Yamasaki, 2001). However, the food consumed by fox species varies during the year due to food availability. For example, in the summer and spring their diet primarily consists of rodents, birds, snakes, rabbits and deer fawns, whereas their food consumption in winter is mostly mice, birds, apples and carrion (Whitaker & Hamilton, 1998).

Red foxes mate between December and February, producing one litter per year after a gestation period of 49–55 days. Cubs are born from March to May, and the average litter size is 3–12 cubs per litter, and usually 4–5 cubs per year in Europe. In areas with a dense fox population, most yearlings do not breed successfully (Larivière & Pasitschniak-Arts,

1996). The reason for this has been demonstrated by studies, which have shown a strong correlation between the number of yearling vixens, which breed and food availability in the area. Cubs are born with light black (grey) fur and weigh about 100g. They are initially blind, with their eyes opening between 10–12 days. The cubs are weaned from 6–8 weeks, and reach maturity at 9–10 months. The vixen stays in the den to provide warmth for the cubs during the first 2 weeks. At this time, the male provides food to the den. The cubs are able to eat solid food after 4–5 weeks of birth, which is provided by both parents (Voigt & Macdonald, 1984; Whitaker & Hamilton, 1998).

The red fox has a typical life span of 3 to 7 years. However, in wild habitats they have been found to live for up to 9 years (Allen & Sargeant, 1993). Their mortality rate can be affected by several factors, such as hunting, trapping, predation and collision with vehicles. In a study conducted in North Dakota, 28% of 363 tagged foxes were shot, 51% were trapped and 21% died for other reasons (Allen & Sargeant, 1993).

1.4.6. Greater white toothed shrew (*Crocidura russula*).

The greater white toothed shrew is a very common species in Eurasia (Burton, 1991). They have a broad distribution throughout different regions of the world including Europe and northern parts of Africa (Burton & Oliver, 1991; Hutterer, 1986; Macdonald & Tattersall, 2001). In the United Kingdom they have been reported in the Herm and Alderney areas (Churchfield, 1988).Furthermore, they occur in the temperate regions which contain high density of insects. The species can inhabit different habitats including woodlands, grasslands and agricultural areas (Duarte, 2003).

They have a medium body size with weight between 11 to 14 g (Balloux *et al.*, 1998). The body length is between 6 to 9 cm including the head while the tail ranges between 3 to 4.6 cm. The dorsal part of the body is reddish brown or greyish in colour whereas the underside is yellowish grey ((Duarte *et al.*, 2003).

The greater white toothed shrew feeds on different species of invertebrate (Burton 1991), and also some vertebrate species including lizards and small rodents (Churchfield, 1988). They build their nest under the stones or burrow (Burton & Oliver, 1991). This species has more reproductive output than the British red toothed shrew, where they produce between four to five litters a year (Macdonald & Tattersall, 2001). Both parents care for their young and defend their territory (Bouteiller-Reuter & Perrin, 2005). The greater

white toothed shrew has a short lifespan up to 18 months in wild habitats (Magnanou, 2009).

1.4.7. Pygmy shrew(*Sorex minutus*)

The pygmy shrew can be found in different parts of the world including Eurasia and Europe. This species is ubiquituous throughout the UK. However, they have been absent from the Isles of Scilly and the Shetlands. They are considered as the only native species present in Ireland (Churchfield, 1988). The population size of this species was estimated at about 8,600,000 in England, 2,300,000 in Scotland, and 1,500,00 live in Wales (Harris, 1995). Furthermore, this species can inhabit different habitats such as forest ,woodland ,grassland and rocky areas (Harris, 1995).

The body size of the pygmy shrew is tiny (Churchfield, 1988). They have greyish brown fur in the upper part of the body. The body length is between 40 to 64 mm, and they weigh between 2.4 to 6.1g, with the tail range between 30 to 46 mm (Macdonald & Tattersall, 2001).

The animal is active during the day and night. The can feed on different species of invertebrates including spiders, woodlice and insect larvae (Macdonald & Tattersall, 2001).In addition to this, the pygmy shrew breeds between April and August, and the gestation period is between 19 to 25 days, with two litter normally produced every year with 4 to 7 young in each litter. They live for up to 16 months (Macdonald & Tattersall, 2001).

1.5. Aims and objectives.

The aims and objectives of this thesis are

- To investigate and characterise arthropod-borne infections in small mammals from both the United Kingdom and Saudi Arabian wildlife. This will be achieved through the analysis of DNA samples from rodents sampled in the UK and Ireland as well as Libyan Jirds and Desert hedgehogs from Saudi Arabia. Characterisation of positive samples through Sanger sequencing will enable the identity if positive samples to be determined. Analyses will assess the importance of individual-level factors, such as sex, age and size, on the probability of hosts being infected.
- To determine the role of red foxes as hosts for vector-borne haemoparasites. This will be through the screening of DNA obtained from red foxes that were collected from the Birstol area following collisions with vehicles.
- To assess the use of next generation sequencing (NGS) methods to investigate arthropod-borne infections. By comparing the bacterial fauna of ticks by both traditional PCR methods and NGS, the pros and cons of each methods will be determined.

This thesis will be structured in the following way: Chapter 2 is a general methods chapter, chapters 3 and 4 report on vector-borne infections of small mammals. Chapter 5 studies the role of red foxes and chapter 6 compares NGS methods with more traditional molecular approaches. Finally, Chapter 7 is a synthesis of the information presented.

Chapter Two

Materials and methods

General Methods.

In this study, molecular techniques were employed to investigate the prevalence and characteristics of different haemoparasites in a variety of wild mammal populations. These included PCR amplification of parasites from the following animal reservoirs: Libyan jirds and desert hedgehogs from Saudi Arabia, bank voles and wood mice from the UK and Ireland and red foxes (*Vulpes vulpes*) from England.

Furthermore, invertebrate tick (nymph stage) species were examined for the presence of haemoparasites using both standard PCR and next generation sequencing (NGS) library techniques.

2.1. Sample collection.

A total of 1138 animal/tick samples were tested for the presence of haemoparasite infections in this study,with the samples having been collected from different countries (Table 2.1).

Table 2.1. Sample types and collection areas.

Host species	Number	Location
Bank vole	5	England
Wood mice	84	England
Red foxes	392	England
Ixodes tick	40	England
Bank vole	133	Ireland
Wood mice	179	Ireland
Pygmy shrew	16	Ireland
Greater white-toothed shrew	56	Ireland
Libyan jird	121	Saudi Arabia
Desert hedgehog	112	Saudi Arabia
Total number	1138	

2.2. DNA extraction.

2.2.1. DNA extraction from rodent and red fox blood samples using a genomic DNA kit.

DNA extraction was performed by using an isolate II Genomic DNA Kit according to the manufacturer's instruction (Bioline, UK). Briefly, 200 µl of blood were placed into 1.5 ml microcentrifuge tubes and 25 µl of proteinase K and 200 µl of Lysis buffer G3 were then added to the samples. The sample was mixed using a vortex for 15 seconds and lysed at 70°C for 10-15 min. 210 µl of ethanol was added to each of the tubes and vortexed for several seconds. The samples were then transferred into an isolate II spin column before being placed in a 2 ml collection tube and centrifuged for 1 minute at 11,6xg (Fisher Scientific/ AccuSpin Micro17). The collection tube was discarded and the isolate II column was placed into a new collection tube, then 500 µl of GW1 buffer was added to the column and centrifuged at 16,000 xg for 1 minute. Again the collection tube was discarded and 600 µl of GW2 was added and centrifuged for 1 minute at 16,000 xg. The flow–through was discarded and any residual ethanol was removed by centrifugation at 16,000 xg for 1 minute. The isolate II column was placed into a 1.5 ml microcentrifuge tube and 100 µl of elution buffer was added to the membrane. The tube was incubated at room temperature for 1 minute before a final centrifugation step was done at 16,000 xg for 1 minute.

2.2.3. DNA extraction from FTA cards for Saudi jirds and hedgehogs.

All jird (121) and hedgehog (112) blood samples used in this study were collected on Whatman FTA cards. The DNA was extracted according to the illustra™ Ready-To-Go™ GenomiPhi™ V3 DNA Amplification Kit protocol. The card was placed into a clean mat and three 2.2.mm circular discs were obtained using a Harris Micro Punch according to the manufacturer's instructions. After each use, the punch was cleaned with filter paper in order to avoid any contamination between samples. The 3 selected discs were placed into a 0.5 ml tube and washed with the FTA purification reagent for 5 min. The FTA purification reagent was then discarded by using a pipette and the same step repeated twice for a total of 3 washes using FTA purification reagent. Next, the selected discs were washed with 200 ml TE buffer (10 mM Tris, 0.1 Mm EDTA, pH 8.0) and incubated for 5 min at room temperature. Again, the TE buffer was discarded using a pipette and the same step was repeated once again for a total of 2 washes. Next, 20 µl of cell lysis buffer was added to

each tube and was incubated on ice for 10 min. After that, 20 µl of neutralization buffer was added with 20 µl of PCR grade water. 10 µl of the previous mixture was taken and 10 µl of denaturation buffer was added to it and transferred to Ready–To-Go Genomiphi cake and the samples were incubated for 2 hours at 30°C. Finally, the samples were heated at 65°C for 10 min and cooled immediately to 4°C.

2.3. Polymerase chain reaction.

2.3.1. Mammalian Tubulin PCR.

Samples were collected from all target hosts and these were checked for DNA validity using mammalian tubulin PCR. PCR was used to confirm that the extracted DNA was suitable for different PCR essays that would then be performed. Reaction involved the amplification of DNA in a 25 µl tube, with the reaction volume containing 2.5 µl of Bioline NH4 PCR buffer, 0.5 µl of forward primer: 5'CGTGAGTGCATCTCCATCCAT'3 (25 pm/µl), 0.5 µl of reverse primer 5'GCCCTCACCCACATACCAGTG'3 (25 pm/µl), 18.75 µl of distilled water, 1 µl of $MgCl_2$ (50 Mm), 0.5 µl of Taq DNA polymerase (5 unit/µl), 0.25 µl of dNTPmix (25mM) and 1 µl of host DNA. For the standard primary reaction one denaturation cycle at 94°C for 5 min was followed by 40 cycles at 94°C for 40 seconds, with annealing at 60°C for 40 seconds and extension at 72°C for 90 seconds with incorporation of a final cycle of extension at 72°C for 10 min.

2.3.2. Polymerase chain reaction (PCR).

A nested polymerase chain reaction was used to amplify a variable region of different haemoparasite DNA including *Trypanosoma*, *Babesia*, *Theileria* and *Bartonella* by using a thermal cycler (Prime elite, Techne). In addition to this a single round of PCR was used to confirm the presence of Candidatus Midchloria in tick samples .The first reaction involved the amplification of DNA in a 25 µl reaction volume consisting of 1 µl of each primer (table 2.2 12.5 µl of 2x My Taq Red mix (Bioline), 8.5 µl ddH2O and 2 µl of DNA template. Both controls (positive and negative) and blank samples were also included to account for contamination and PCR errors. Different PCR programs were set for different organisms (Table 2.2).

All PCR programs were started with an initial denaturation step at 94°C for 5 min and finished with one final extension cycle 72°C for 5 min followed by different temperatures when denaturation, annealing and extension stage were used (Table 2.3). The second cycle of nested PCR was conducted also in a 25 µl reaction volume containing 1 µl of each of the primers, 12.5 µl of Master Mix, 9.5 µl ddH2O and 1 µl of the PCR products from the first PCR reaction as DNA template, followed by the same PCR program that was used in the first round. Once the PCR had been performed, the samples were separated using 1.5% agarose gel in 100 ml of TBE buffer and 3 µl GelRed (Bioline, UK). 10 µl of each of the PCR products were loaded into each gel well, and the gel was run for one hour at 110 V, before the gel was visualized on a UV trans-illuminator. A 1 kb and 100 pb hyperladder (Bioline, UK) were used as a marker size in each gel.

Table 2.2. Target gene, oligonucleotide sequence, and product size for different haemoparsites.

Infection	Target gene	Primer (10pmol/ µl)	Oligonucleotide sequence (5'→3')	Product size	Reaction	Reference
Trypanosoma	18SrRNA	TRP927F	GAAACAAGAAACACGGGAG	400 bp	1st round	(Noyes *et al.*, 2002)
		TRP927R	CTACTGGGCAGCTTGGA			
		SSU561F	TGGGATAACAAAGGAGCA		2nd round	
		SSU561R	CTGAGACTGTAACCTCAAAGC			
Babesia	Beta tubulin	F34	TGTGGTAACCAGYGGWGCCAA	200 bp	1st round	(Cacciò *et al.*, 2000)
		R323	TCNGTRTARTGNCCYTTRGCCCA			
		F79	GARCAYGGNATNGAYCCNGTAA		2nd round	
		R206	ACDGARTCCATGGTDCCNGGYT			
Theileria	18SrRNA	BMF1	GCGATGTATCATTCAAGTTTCTG	600 bp	1st round	Simpson et al.,2005
		BMR1	TGTTATTGCCT TACACTTCCTTGC			
		BMF2	ACGGCTACCAC ATCTAAGGAAGGC		2nd round	
		BMR2	TCTCTCAAGGTGCTGAAGGA			

Bartonella	Citrate synthase gene (gltA)	443F	GCTATGTCTGCATTCTATCA	400 bp	1st round	(Birtles & Raoult, 1996; Norman *et al.*, 1995)
		1137R	AATGCAAAAAGAACAGTAAACA			
		781F	GGGGACCAGCTCATGGTGG		2nd round	
		1137R	AATGCAAAAAGAACAGTAAACA			
Candidatus Midchloria	16SrRNA	Midi F	GTACATGGGAATCTACCTTGC	700bp	1st round	(Cafiso *et al.*, 2016)
		Midi R	ATCCCAACATATAGCACTCAT			

Table 2.3. Different PCR cycling conditions for different haemoparasites.

Infection	Number of cycles	Denaturation	Annealing	Extension	References
Trypanosoma	30	94°C for 30s	55°C for 60s	72°C for 90s	(Noyes *et al.*, 2002)
Babesia	35	94°C for 30s	55°C for 30s	72 °C for 60s	(Cacciò *et al.*, 2000)
Theileria	35	95°C for 10s	50°C for 20s	72°C for 50s	Simpson *et al*.,2005
Bartonella	35	95°C for 10s	50°C for 20s	72°C for 50s	(Birtles & Raoult, 1996; Norman *et al.*, 1995)
Candidatus Midchloria	35	95°C for 45s	57°C for 45s	72°C for 60s	Cafiso *et al*., 2016)

2.3.3. Real time PCR.

Real time PCR was used to confirm the presence of *Anaplasma ,Borrelia* and *Rickettsia* within the collected ticks .The PCR mixture was include 1 µl of each of the primers (2.4), 10 µl of My Taq mix (Bioline), 5 µl ddH2O and 1 µl of the probes and 2 µl of DNA. The cycling condition for each infection is described in the table 2.5.

Table 2.4. Target gene, oligonucleotide sequence and probes for different bacterial samples.

Infection	**Target gene**	**Primer(10pmol / µl**	**Oligonucleotide sequence (5'→3')**	**References**
Borrelia	16SRNA	Bb23SF	CGAGTCTTAAAAGGGCGATTTAGT	(Courtney *et al.*, 2004)
		Bb23SR	GCTTCAGCCTGGCCATAAATAG	
		Bb23Sp-FAM	AGATGTGGTAGACCCGAAGCCGAGTG	
Anaplasma	16SrRNA	APMS2F	ATGGAAGGTAGTGTTGGTTATGGTATT	(Courtney *et al.*, 2004)
		APMS2R	TTGGTCTTGAAGCGCTCGTA	
		APMS2 probe	TGGTGCCAGGGTTGAGCTTGAGATTG	
Rickettsia	gltA	RKND03F	GTGAATGAAAGATTACACTATTTAT	(Socolovschi *et al.*, 2010)
		RKND03R	GTATCTTAGCAATCATTCTAATAGC	
		RKND03 probe	CTATTATGCTTGCGGCTGTCGGTTC	

Table 2.5 .The condition cycles for *Borrelia, Anaplasma* and *Rickettsia* infection.

Infection	Number of cycles	Denaturation	Annealing	References
Borrelia	40	95°C for 15s	57°C for 60s	(Courtney *et al.*, 2004)
Anaplasma	40	95°C for 15s	57°C for 60s	(Courtney *et al.*, 2004)
Rickettsia	40	92°C for 1s	60°C for 35s	(Socolovschi *et al.*, 2010)

2.4. Gel electrophoresis.

Gel electrophoresis was used to visualize the PCR products. To make a 1.5% gel, 1.5g of agarose powder was placed into a duran flask and 100 ml of 1x TBE buffer was added. The bottle was microwaved for about 30 seconds at the maximum power. The mixture was then swirled and re-heated for 30 seconds until the agarose powder had fully dissolved. The bottle was then put into a shaker and when the mixture had cooled to approximately 50°C, 3 µL of Gel Red was added to the mixture and the liquid was swilled gently. The mixture was then poured into a gel casting tray using appropriate dams and combs and the gel was then left to set for 20-30 min. Then the dams and comb were removed and 10 µL of each PCR product was loaded into the wells. The PCR product was allowed to electrophorese at 100 volts for 1 hour. The gel was then visualized using a UV trans-illuminator in order to check for the correct size of the amplified gene. Visualized images were then saved on the computer.

2.5. Purification of PCR products.

PCR product was purified using a Bioline Isolated II PCR kit. One volume of PCR product was mixed with 2 volumes of CB binding buffer. Isolated II PCR column was placed into 2 ml collection tubes and then the sample was centrifuged at 11.6 xg (Fisher Scientific/AccuSpin Micro17) for 30 seconds. Flow–through was discarded from the collection tube and 700 µL of CW washing buffer was added to the PCR column and the samples were centrifuged at 11.6 xg (Fisher Scientific/AccuSpin Micro17) for 30 seconds.

The same step was repeated in order to minimize chaotropic salt carry over. The flow through was then discarded and the sample was centrifuged again to remove any CW washing buffer material from the previous step. The isolated PCR column then was placed into a 1.5ml tube and 25 µl of C elution buffer was added; the sample was then incubated at room temperature for 1 minute and then centrifuged for 1 minute at 11000 rpm. The purified PCR products were stored at -20°C.

2.6. DNA concentration.

The DNA concentration of all positive samples was measured with a Nanodrop spectrophotometer (Thermo fisher scientific). To begin with, 1 µl of distilled water was placed into the lower optical surface and the upper arm in order to confirm the blank measurement by selecting blank measurement on the screen. Once the blank measurement had been taken, both optical surfaces were cleaned by a clean lab wipe. Next, 1 µl of nucleic acid sample was placed into the lower optical surface and we ensured that the upper arm was in contact with the DNA sample and selected the most appopriate measurement on the screen. Then, the software automatically calculated the nucleic acid concentration and purity ration 260/280 nm of the sample.

2.7. Bioinformatics software.

Several computer software and statistical tools have been used in this study to analyse our data. These are described as follows.

2.7.1. Finish TV.

Finish TV is computational software (http://officialsite.pp.ua/?p=2958497), which can assist the viewing and analysis of DNA sequences once they have been received by the Bioscience Sequence Company. The software can display raw DNA sequences and was used to analyse the DNA sequence in this study.

2.7.2. Blast Tool.

The blast tool is a software program provided by NCBI (of PubMed fame) and has been used to check our DNA sequence with best-hit sequence in the genBank. The program is available at https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome.

2.7.3. Clustal omega.

Clustal omega is the latest software that can align more than one DNA sequence sample together in an accurate manner. The program will assist the operator in highlighting the most similar and conserved region between those sequences and it has been considered an important program for polygenetic analysis. The program is available at http://www.ebi.ac.uk/Tools/msa/clustalo/.

2.7.4. Statistical analysis.

Chi square was performed by Minitab 16 software (Minitab Inc, USA) in order to look for associations between infections and host factors such as sex, age, body condition and seasonal collection. The results were considered to be significant when $P<0.05$.

2.7.5. Phylogenetic tree.

A phylogenetic tree of the evolutionary relationship between detected species in the study was done using MEGA, version 6 (Tamura *et al.*, 2013) in order to confirm our results. Species of parasites that were deposited in GenBank were compared with our species, and all sequences were aligned using Clustal W2 (Larkin *et al.*, 2007) and phylogenetic trees were constructed by using the neighborhood joining test (Saitou & Nei, 1987).

2.8. Next generation sequencing for tick samples.

2.8.1. DNA extraction.

Forty tick samples were investigated for the presence of microbial and parasitic infections by amplifying and sequencing regions of the 16S rRNA and 18S rRNA, respectively. Tick DNA was extracted according to a modified ZR-Duet™ DNA/RNA MiniPrep Plus (Zymo Research) manufacturer's instructions, using bead beating plus an additional cleanup step from a ZR- Insect DNA Extraction kit (Zymo Research). Briefly, 400 µl of DNA/RNA shield, 40 µl of digestion buffer (PK), 20 µl proteinase K, ticks were placed in ZR Bashing Bead Lysis tube and secured in Qiagen TissueLyser II bead beater, which is fitted with 2 ml tube holder and run for 10 min at the maximum speed, then centrifuged for 1 minute at 16,000 x g. Supernatant (~ 350 µl) was transferred to a new tube and an equal amount of DNA/RNA lysis buffer was added. The total amount was transferred to Zymo-spin IV spin filter and collection tube, samples were centrifuged for 1 minute at 16,000 x g and the flow-through (~ 400 µl) was transferred to a Zymo IIc filter, which was also placed in the collection tube and centrifuged for 30 seconds at 16,000 x g and placed into a new collection tub to commence the washing steps, 400 µl of DNA/RNA prep buffer were added and centrifuged at 16,000 x g for 30 seconds and the flow-through was discarded. In the following step, 700 µl of DNA/RNA wash buffer was added to the tube and it was centrifuged for 30 seconds at 16,000 x g, and the flow-through was once again discarded. In the next stage, 400 µl of DNA/RNA wash buffer was added into the column and centrifuged at 16,000 x g for 2 mins to remove residual wash buffer. Subsequently, the column was placed into a clean microcentrifuge tube and 100 µl of DNase /RNase–free water was added. Finally, the sample was incubated at room temperature for 5 min and then centrifuged for 30 second at 16,000 xg to elute the DNA from the respective column.

2.8.2. 16S rRNA Amplicon PCR conditions.

The V4 region of the 16S rRNA gene was targeted for microbial community analysis, following protocols from the Earth Microbiome Project {http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/}. Reactions consisted of 12.5 µl 2 x KAPA HiFi HotStart Ready Mix, 5 µl of forward primer (515), 5 µl of reverse primer (806) and 2.5 µl of genomic DNA. PCR reaction was started with one cycle of denaturation at 95°C for 3 min, followed by 25 cycles at 95°C for 30 seconds,

annealing at 55°C for 30 seconds and an extension cycle was performed at 72°C for 30 seconds with incorporation of a final cycle of extension at 72°C for 5 min.

Table 2.6. Target gene and oligonucleotide sequence different bacterial infection.

Target gene	**Primer(10pmol/ µl**	**Oligonucleotide sequence (5'→3')**	**Size of gene/ bp**	**Reference**
16SrRNA	515F	GTGYCAGCMGCCGCGGTAA	300 bp	(Caporaso *et al.*, 2011)
	806R	GGACTACNVGGGTWTCTAAT		

2.8.3. First PCR Clean-up.

The amplicon PCR plate that contained the PCR products was centrifuged for 1 minute at 1,000 x g. the AMPure XP beads (Beckman Coulter, High Wycombe, UK) were incubated for 20 minutes at room temperature and then they were vortexed for 30 seconds in order to confirm that all beads were dispersed. 20 µl AMPure beads were added to each well with gentle pipetting up and down for 10 times using a multichannel pipette. The plate was then vortexed for 2 minutes at 0.3xg and incubated at room temperature for 5 minutes without shaking. Once that had finished, the plate was placed on the magnetic stand for 2 minutes, and then the supernatant was discarded as the plate remained on the magnetic stand. After that, the plate containing the beads on the magnetic stand was washed using 200 µl freshly prepared 80% Molecular Grade ethanol, which was added to each well and incubated for 30 seconds, and the supernatant was discarded, before repeating the first wash step for a second time. Following this, the beads were dried for 10 minutes on the plate on the magnetic stand. The PCR plate was removed from the magnetic stand and added 52.5 µl of 10mM Tris-HCl pH8.5 into each well with gentle up and down pipetting. The PCR plate was then vortexed for 2 minutes to ensure that the mixture was fully resuspended. Two minutes of incubation at room temperature occurred before the PCR plate was placed again on the magnetic stand for 2 minutes. The final step was to transfer 50 µl of the supernatant to a new 96-well PCR plate.

2.8.4. Labelling of purified PCR products.

Illumina sequencing adapters and dual indexes were attached to the purified PCR products by using the Nextera XT index kit (Illumina) in a 96 well, plate format (Figure 2.1). tubes that contain index primer (orange caps) were placed horizontally from column 1 to 12 of the index kit while the tubes that contain the index 2 primer (white caps) were placed vertically from from A to H of 96 PCR plate (Figure 2.1).

Index PCR was performed according to the manufacturer's instructions, consisting of 25 µl 2x KAPA HiFi HotStart Ready Mix (Kapa Biosystems), 5 µl of index 1 primer, 5 µl of index 2 primer (Table 2.7) and 5 µl of genomic DNA as in table 2.3. PCR reaction was conducted with one cycle of denaturation at 95°C for 3 min, followed by 8 cycles at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension cycle was performed at 72°C for 30 seconds with incorporation of final cycle of extension at 72°C for 5 min.

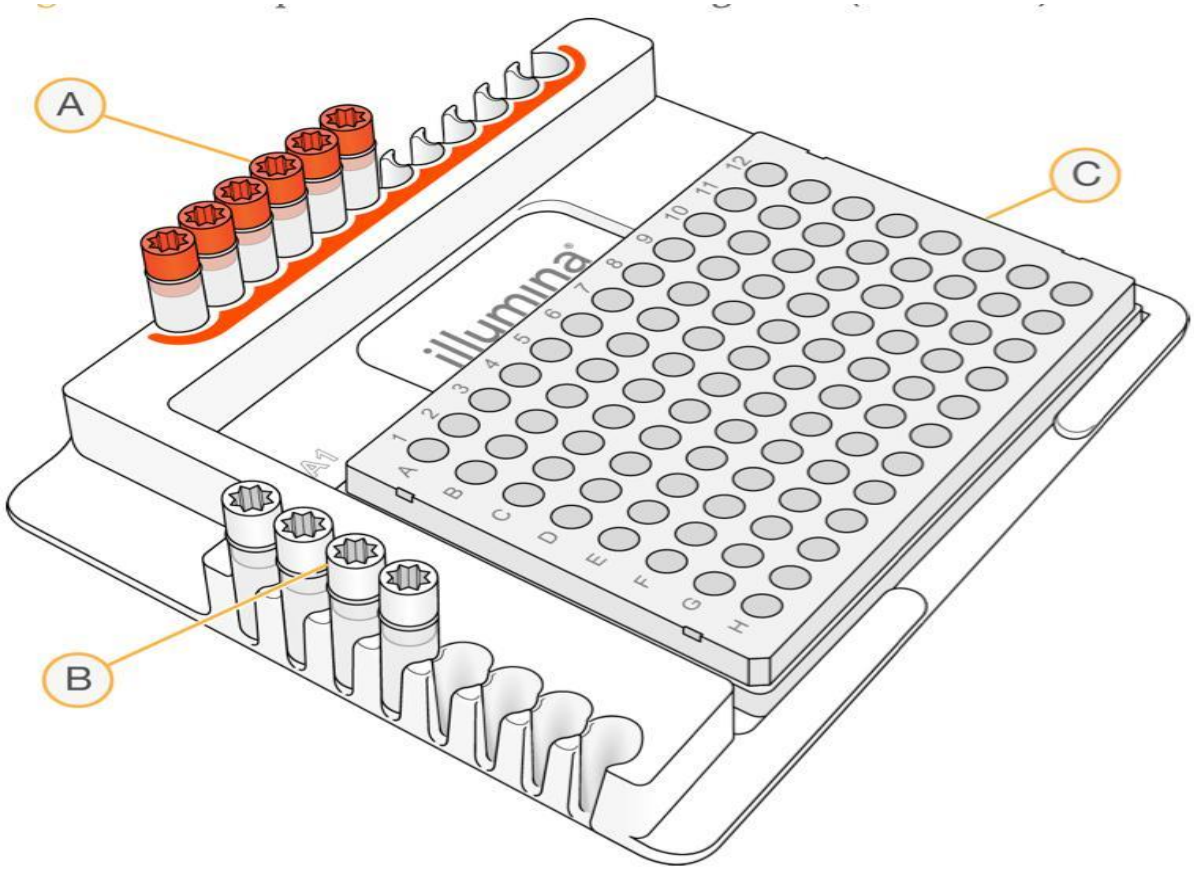


Figure 2.1. TruSeq Index PCR plate, A. Index 1 primer (orange caps), B. Index 2 (white plate), C. 96- well plate.

Table 2.7. The dual indexing primers used for individual sample bioinformatic demultiplexing from mixed pools, to allow for high-throughput sequencing. Each sample gets one primer from index 1 and another primer from index 2.

Gene of interest	**Index 1**	**Sequence**	**Index 2**	**Sequence**
16S rRNA	N701-A	TAAGGCGA	S513-C	TCGACTAG
	N702-A	CGTACTAG	S515-C	TTCTAGCT
	N703-A	AGGCAGAA	S516-C	CCTAGAGT
	N704-A	TCCTGAGC	S517-C	GCGTAAGA
	N705-A	GGACTCCT	S518-C	CTATTAAG
	N706-A	TAGGCATG	S520-C	AAGGCTAT
			S521-C	GAGCCTTA
			S522-C	TTATGCGA

2.8.5. Purification of labelled 16SrRNA libraries (second clean up).

The second clean-up was performed in the same way as the first with two exceptions. 1. 56 µl of AMPure XP beads were added to each well instead of adding 20 µl, and 2. Following washing, DNA was eluted in 27.5 µl rather than 52.5 µl of 10mM Tris pH8.5, with 25 µl final supernatant volume transferred from every well to a new 96-well plate.

2.8.6. Quantification of 16S rRNA libraries.

DNA concentration was measured using the Qubit dsDNA HS (High Sensitivity) Assay according to the manufacturer's instructions. The assay consists of several components such as Qubit dsDNA HS Reagent (Component A), Qubit dsDNA HS buffer (Component B), Qubit dsDNA HS Standard (Nowak, 1999)1 (component C) and Qubit dsDNA HS Standard#2 (Component D). The protocol was started using 0.5 ml PCR tubes, which were labeled on the tube lids as samples and standers. The Qubit working solution was prepared by diluting the Qubit dsDNA HS Reagent (Component A) 1:200 in Qubit dsDNA HS

buffer (Component B) using a new plastic tube. 198 µL of the Qubit working solution was added to each 0.5ml PCR tube that contained 2 µl of sample DNA. Qubit Standard 1 tube contained 190 µl of the Qubit working solution and 10 µL was taken from Qubit dsDNA HS Standard #1 (0 ng/µl; Component C). Qubit standard 2 tube contained 190 µL of the Qubit working solution and 10 µL of the Qubit dsDNA HS Standard #2 (10ng/µl; Component D) with a total volume of 200ul was made up for each tube. The tubes were then vortexed for 2-3 seconds and they were incubated for 2 minutes at room temperature. The tubes were taken to the machine and dsDNA High sensitivity was selected on the screen, followed by selection of the Read Standard functionality on the screen. Standard 1 was placed into the chamber and the lid was closed followed by pressing Read. The same steps were done for Standard 2. Once that had been done, we ran the samples. The sample volume units were selected and each of the individual samples were placed into the sample chamber with the lid closed before the tube was read. The reading was completed in 3 seconds, and then other samples were read.

Samples were pooled in an equimolar fashion using Qubit readings wherever possible. In cases where the measurement was too low, then an arbitrary lower value equivalent to the next lowest sample was assigned to the sample for inclusion in the pool (including, particularly, negative controls).

2.8.7. Library Denaturing and Mi-seq sample loading.

NaOH (Fisher Scientific, UK) was used in order to denature the pooled libraries .Furthermore, hybridization buffer was used for dilution and heating of denatured libraries as stage to prepare for the generation of cluster and data sequencing. MiSeqv3 reagent kit was used as recommended by Illumina in order to improve run metrics.

2.8.7.1. Denaturation and dilution of DNA.

A standard normalization was done according to the denaturation and diluted library guide. The pooled library was diluted into the 4nM by adding 1 µl of pooled library and 5.6 µl water to obtain 6.5 µl of the diluted library.

Fresh (1M) NaOH was obtained in Falcon tube by adding 2 grams of Sodium hydroxide thah mixed with 50 ml of water. The fresh 1M NaOH was diluted into 0.2M by adding 8ml of water into 2ml of 1M NaOH.

2.8.7.2. Denaturation of diluted library.

A combination of 5 µl of 4 nM pooled library was combined with 5 µl of 0.2 N NaOH in 1.7 ml tube and centrifuged for 1 min at 280 xg and incubated at room temperature for 5 min in order to obtain single strand of the DNA, 990 µl of pre-chilled HT1 was added and the mixture 20 Pm was placed on ice until final dilution prepared. A final concentration (10 pM) of denatured and diluted DNA was prepared by adding of 20 Pm (500 µl) denatured library and Pre-chilled HT1 (500µl). The samples were mixed, centrifuged and kept on ice.

2.8.7.3 .Denaturation and dilution of PhiX control and Amplicon Library combination.

The denaturation and dilution of PhiX (control) 4 nM PhiX was prepared by adding 2 µl of 10 nM PhiX library in tube that contained 3 µl of 10mM Tris pH 8.5 ,and 5 µl of 0.2 N NaOH was added, vortexed and incubated at room temperature for 5 min, after that a single strand of denatured PhiX was obtained . The 20 pM PhiX library were prepared by mixing 10 µl of denatured PhiX library with 990 µl of pre-chilled HT1, and 1:1 dilution was performed, 90 µl of PhiX control (diluted sample) was added to 510 µl of diluted amplicon library, combined sample was placed on ice and loaded into MiSeqV3 reagent cartridge.

2.9. Bioinformatics analysis.

The bioinformatics analysis was kindly provided by Dr.Ian Goodhead. Reads were demultiplexed and adapter trimmed using CASAVA 1.8 (Illumina). DADA2 v1.4 (Callahan *et al.,* 2016) was used to process the processed sequencing data: PhiX was removed, reads were truncated to 2x240bp, allowing zero N's, truncating quality scores less than two and removing sequences with a maximum expected error of two (truncLen=c(240,240), maxEE=2, truncQ=2, maxN=0, rm.phix=TRUE). Sequences were dereplicated and chimeric sequences removed. Clustering and taxonomy assignment was then performed with DADA2, assigning taxonomy with the Silva v128 database at 97% identity.

Chapter Three

Comparison of vector-borne protozoan infections in rodents from England and Ireland: the effect of invasion.

3.1. Introduction.

The invasion of new species of host and parasites in many areas can lead to changes in community structure and biodiversity loss (McGeoch *et al.*, 2010).The full understanding of those factors which have impact on native species becomes more interesting and important as the number of introduced species increased. The parasites may play important roles, either as invasive species or by mediating interactions between other community members. Moreover, large numbers of studies have shown that parasites can make changes in the host community with the respect to abundance (Chen *et al.*, 2011; Lafferty *et al.*, 2008) showing the potential importance of host parasite interactions at different levels of the population. Also, the parasites dynamics can by influenced by population interaction and structure. In this study the different haemoparasitic infections such as *Trypanosoma* spp, *Babesia* spp and *Thieleria* spp have been investigated in a sample of small mammals that been collected from different areas in United Kingdom and Ireland. Furthermore, this study examined the impact of the invasion host (bank vole in Ireland) on the prevalence of infection in native species (wood mice in Ireland). A small number of bank voles are thought to have been introduced from Germany to Ireland in 1926 (Stuart *et al.*, 2007). Recently, some studies have shown that bank voles occurred in an area representing 33% of the south-west of Ireland (Montgomery *et al.*, 2012; White *et al.*, 2012). This study will demonstrate the potential impact of invasive species on the host-parasite relationships of native species. By sampling sites across an invasion front in Ireland, the influence of the introduced bank vole on the epidemiology of infections caused by flea-transmitted haemoparasites of the genus Bartonella in native wood mice (*Apodemus sylvaticus*) was evaluated (Telfer *et al.*, 2005). Bank vole introduction has affected the wood mouse-Bartonella interaction, with the infection prevalence of both *Bartonella birtlesii* and *Bartonella taylorii* declining significantly with increasing bank vole density. The results are consistent with the 'dilution effect' hypothesis.

In the UK, British wild mammals have been demonstrated to host different haemoparasitic infections such as *Babesia, Trypanosomes and Bartonella.* These parasite species were detected in both populations of bank vole and wood mice in the UK (Bown et al., 2008; Bown, Bennett, & Begon, 2004; Noyes et al., 2002). The genus *Trypanosoma Herpetosoma* has been reported in many countries around the world and fleas are considered the most important vectors. Rodents become infected by trypanosome species through the following two main routes: through contamination of a bite or at the feeding

site by *metatrypanosoma* when the organism is shed in flea faeces. The other mechanism is by ingestion of a contaminated flea during grooming. Through this second mode of infection, the trypanosome develops into the infective *metatrypanosoma* stage and then penetrates the oral mucous membrane to reach the bloodstream (Smith *et al.*, 2005). Different flea species can transmit the parasite this way. For example, *T. lewisi* is transmitted by *Nosopsyllus fasciatus* (northern rat flea), which is an important vector for this pathogen known to infect a wide range of rat species (Molyneux, 1969b).

Babesiosisis a tick- borne disease of human and animal populations. Human babesiosis is caused by different species of *Babesia* such as *B. divergens* and *B. microti* (Gorenflot *et al.*, 1998). Animal species such as cattle and rodents are considered to be reservoir hosts for *B. divergens* and *B. microti* (Homer *et al.*, 2000). The occurrence of *Babesia* species among small mammals has been investigated in different parts of Europe. A recent study that was conducted in Slovakia showed that the presence of *B. microti* was investigated among several rodent species, and a relativilly high prevalence of infection was noted at 40% in *Microtus agrestis* followed by 2.2% and 0.4% in mice and bank voles (Hamšíková *et al.*, 2016). In England, the prevalence of infection from field voles and shrews was reported to be 30.4% and 30.3%, respectively (Bown et al., 2011).

While the prevalence of *B. microti* is relatively high in rodent populations (Siński *et al.*, 2006),the same cannot be said for *Theileria* spp in the aforementioned mammals. Indeed, a thorough literature review identified a small number of studies, which highlight the rarity of this organism in the rodent population. Wolf and colleagues performed a study in Brazil and examined 42 rodents, where three rodents (*Thrichomys pachyurus*) were positive (prevalence of 7%), for new piroplasmid genotypes, phylogenetically (18S rDNA gene) related to *Theileria bicornis* (Wolf *et al.*, 2016). This particular species has only been identified in rhinoceros on the African mainland and is therefore a very surprising finding (Otiende *et al.*, 2015). The remaining exception to the study by Wolf and colleagues is the identification of *Theileria youngi*, which was identified in 61% of Californian dusky-footed woodrats (Kjemtrup *et al.*, 2001).

Aims

The aims of this experiment were to investigate the prevalence and species of *Trypanosomes, Babesia* and *Theileria* infection in small mammals collected from a number of disparate UK and Ireland regions.

- Identify the prevalence of infection in different small mammals in the UK and Ireland regions.
- Characterise the detected parasites and doing phylogenetic tree to compare them with other parasites which found in the world to explain the evolution history.
- Determine the potential impact of invasive species on the host-parasite relationships of native species.

  .

3.2. Methods

Samples of DNA extracts from Ireland were provided by Dr Alan Harrison and Professor Ian Montgomery of Queen's University Belfast. The samples were collected during fieldwork conducted by them during 2010/11 as part of a study investigating the spread of invasive small mammals in Ireland (Montgomery et al., 2012). Animals were humanely euthanised and DNA extracted from liver using a Qiagen DNAeasy Tissue Kit (Qiagen, UK). DNA extraction from rodent samples that been collected from 3 sites in the UK was performed using a previously described protocol in Chapter 2.2.1. A nested PCR was performed to detect the presence of *Trypanosoma Babesia* and *Theileria* infections (Table 2.2 and 2.3 in Chapter 2). PCR products were visualized using gel electrophoresis in order obtain the correct band size, which could be used to identify the presence of different infected species using specific primers. The positive samples were purified and sent for sequencing to Source Bioscience as was previously described in chapters 2.5 and 2.6, respectively. The sequences were analyzed by using different bioinformatic software programs including Finish TV (2.7.1), Blast Tool (2.7.2) and Clustal omega (2.7.3.). Based on the multi -alignment analysis, MAGA 6 program was applied for phylogenetic analysis to identify the relationship between different species of trypanosoma and a phylogenetic tree was constructed. The bootstrap confidence intervals of the phylogenetic tree were based on 1000 replicates.

3.3. Results

A total of 473 animals were trapped and blood and liver samples were taken; 89 samples from England (5 bank voles, 84 wood mice), while 384 samples were from Ireland (133 bank voles, 179 wood mice, 16 Pygmy Shrews and 56 Greater white-toothed shrews) as shown in Table 3.1.

Table 3.1. The total number of tested samples from different locations.

Mammal species	**Number of tested samples**	**Location**
Bank vole	5	MalhamTarn, UK.
Wood mice	13	MalhamTarn, UK.
Wood mice	51	Salford, UK
Wood mice	20	Accrington, UK
Bank vole	133	Ireland
Wood mice	179	Ireland
Pygmy shrew	16	Ireland
Greater white-toothed shrew	56	Ireland

3.3.1. Mammalian tubulin gene amplification.

The tubulin gene was amplified (1000 bp) from rodent samples and the results illustrate that our samples were suitable for further assay (Figure 3.1).

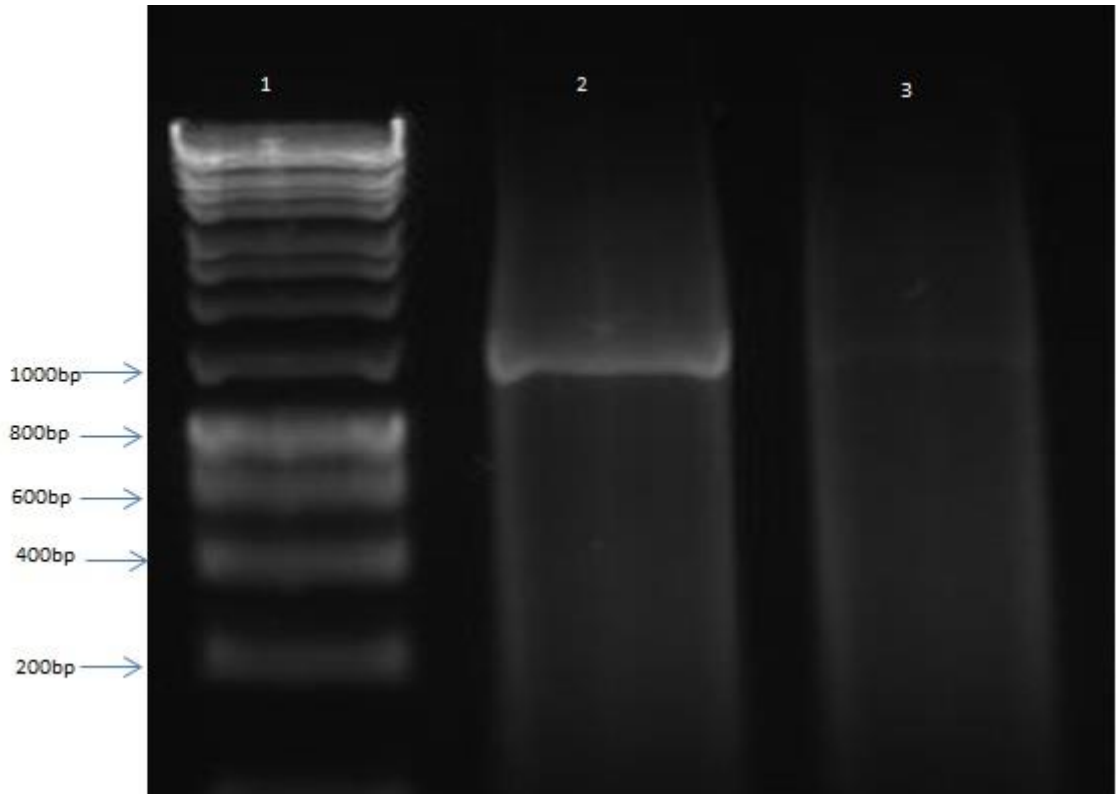


Figure 3.1. Figure illustrates PCR product size of beta tubulin gene from rodent sample. Lane 1 represents the molecular weight marker (1 kp). Lane 2 is the loaded sample. Lane 3 is the negative control. Band in lane 2 indicates the expected size of the gene (1000 bp).

3.3.2. Haemoparasitic infections in different species of mammals.

This study was able to confirm only the presence of *Trypanosoma* spp among collected samples from the United Kingdom and Ireland. The detected species were found in bank vole and wood mice population from the UK and Ireland respectively (Table 3.2)

Table 3.2. The total number of positive samples for *Trypanosoma* spp infections.

Host	**Number of animal**	**Positive sample of *Trypanosoma***	**Location**
Bank vole	5	1	Malham Tarn, UK
Wood mice	13	0	Malham Tarn, UK
Wood mice	51	0	Salford, UK
Wood mice	20	0	Accrington, UK
Bank vole	133	0	Ireland
Wood mice a*	93	0	Ireland
Wood mice b*	86	3	Ireland
Pygmy shrew	16	0	Ireland
Greater white-toothed shrew	56	0	Ireland

a*, area where wood mice and bank vole present ,b*, area where only wood mice present

3.3.2.1. *Trypanosoma* spp infection.

A total of 138 bank voles, 263 wood mice, 16 Pygmy shrews and 56 Greater white-toothed shrews were investigated for the presence of *Trypanosoma, Babesia,* and *Theileria* DNA by amplification of the18S rRNA. None of the samples tested positive for *Babesia* spp or *Theileria* spp, whilst 1/138 (0.7%) bank voles and 3/263 (1%) wood mice were positive for *Trypanosoma* infection based on the expected 490 bp product (Figure 3.2). In this study, *Trypanosoma* spp were only detected in bank vole and wood mice from the UK and Ireland respectively. Furthermore, there was no evidence of infection in wood mice that live in sympatry with bank voles. However, there was no significant difference in prevalence of infection in wood mice in areas where bank voles were present compared to those sampled in areas beyond the bank vole invasion range (Fishers Exact test, P= 0.1089). The positive PCR products from both hosts were sequenced and aligned with other *Trypanosoma* spp 18S rRNA gene sequences in GenBank, and the obtained sequence from bank voles was most to be similar to *T. evotomys* (Figure 4.3) which is believed to be host-specific for the bank vole. Furthermore, the sequence result was 100% similar to *T. evotomys* (Ay043356.1), which was deposited in the GenBank database (Table 3.2). The positive samples from wood mice were analysed and obtained sequences were identified as *T. grosi* (Figure 4. 4) (AB175624.1) (Table 3.3).

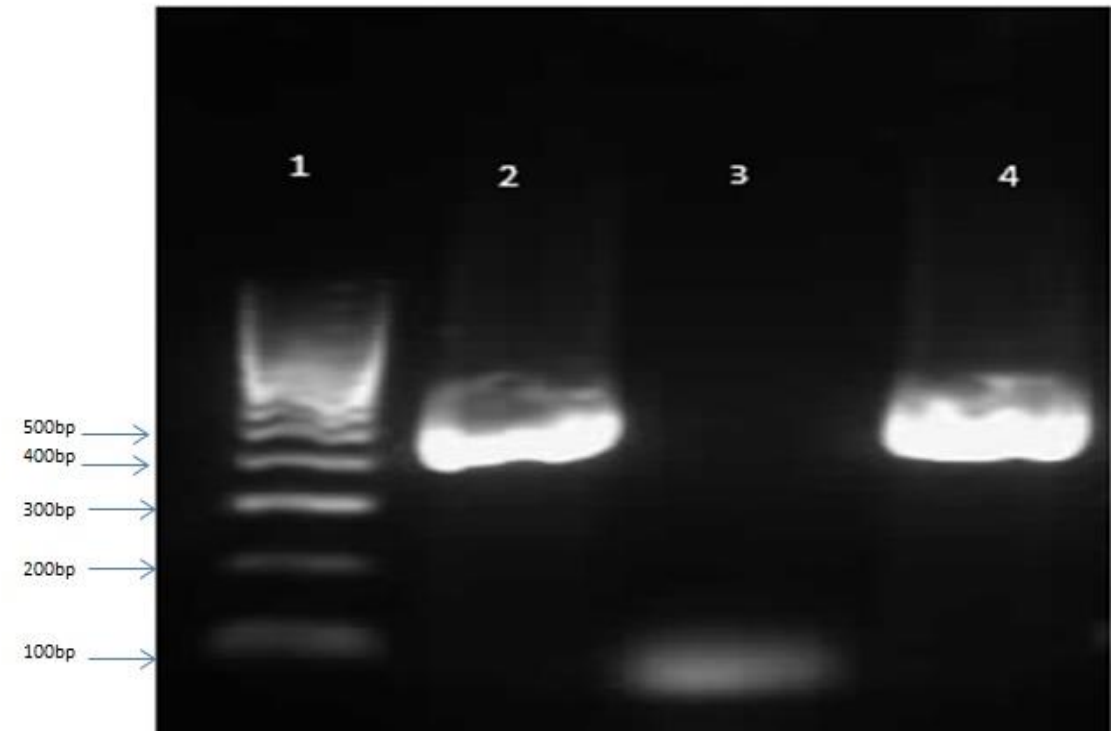


Figure 3.2. 1.5% agarose gel image showing the detected 18S rRNA gene of trypanosomes in rodent samples. Lane 1, represents the marker (100 bp). Lane 2, wood mice (*T.grosi*) sample. Lane 3, a negative control. Lane 4: positive control for *T. evotomys.*

Alignment between the obtained sequences from infected rodents with the closest related species in genBank database are presented (Figures 3.3 and 3.4).

```
CLUSTAL O(1.2.4) multiple sequence alignment

Bankvolesamples      ----------------------------------------GTCTATTGGAGATTATGGGGC
AY043356.1           GCCTATAGGCCACCGTTTCGGCTTTTGTTGGTTTTAACAGTCTATTGGAGATTATGGGGC
                                                            *********************

Bankvolesamples      TGTGCGACAAGCGCTCGAGTGCTCTCCTTTTCGGTGACGCTCGGCGCCTTTGTGGGAAAT
AY043356.1           TGTGCGACAAGCGCTCGAGTGCTCTCCTTTTCGGTGACGCTCGGCGCCTTTGTGGGAAAT
                     ************************************************************

Bankvolesamples      CCACGGTTGCCTCGGCAGGCTTCGGTCTCGCGGAGAGCATTTCCGTCTTCCCTCAACTCG
AY043356.1           CCACGGTTGCCTCGGCAGGCTTCGGTCTCGCGGAGAGCATTTCCGTCTTCCCTCAACTCG
                     ************************************************************

Bankvolesamples      CGGCATCCAGGAATGAAGGAGGGTAGTTCGGGGGAGAACGTACTGGTGCGTCAGAGGTGA
AY043356.1           CGGCATCCAGGAATGAAGGAGGGTAGTTCGGGGGAGAACGTACTGGTGCGTCAGAGGTGA
                     ************************************************************

Bankvolesamples      AATTCTTAGACCGCACCAAGACGAACTACAGCGAAGGCATTCTTCAAGGATACCTTCCTC
AY043356.1           AATTCTTAGACCGCACCAAGACGAACTACAGCGAAGGCATTCTTCAAGGATACCTTCCTC
                     ************************************************************

Bankvolesamples      AATCAAGAACCAAAGTGTGGGGATCGAAGATGATTAGAGACCATTGTAGTCCACACTGCA
AY043356.1           AATCAAGAACCAAAGTGTGGGGATCGAAGATGATTAGAGACCATTGTAGTCCACACTGCA
                     ************************************************************

Bankvolesamples      AACGATGACACCCATGAATTGGGGAGTTTTTGGTCGTAGGCGGGGTCGGGTTCATCTCGC
AY043356.1           AACGATGACACCCATGAATTGGGGAGTTTTTGGTCGTAGGCGGGGTCGGGTTCATCTCGC
                     ************************************************************

Bankvolesamples      TCCTCGTCTCGCCAATGTGTATCAATTTACGTGCATATTCTTTTTGGTCCTCGCAAGGGG
AY043356.1           TCCTCGTCTCGCCAATGTGTATCAATTTACGTGCATATTCTTTTTGGTCCTCGCAAGGGG
                     ************************************************************

Bankvolesamples      TCCTTTTTACGGGAATATCCTCAGCACGTTATCTGACTTCTTCACGCGAAA
AY043356.1           TCCTTTTTACGGGAATATCCTCAGCACGTTATCTGACTTCTTCACGCGAAA
                     ***************************************************
```

Figure 3.3**.** Clustal W alignment for sequence of 18S rRNA gene detected from bank vole with fragment of the 18S rRNA gene from *Trypanosoma evotomys* deposited in GenBank (accession number AY043356.1).

```
woodmicesample      ------------------------------------------------------------TCTA
AB175624.1          GGATAACAAAGGAGCAGCCTATAGGCCACCGTTTCGGCTTTTGTTGGTTTTAACAGTCTA
                                                                               ****

woodmicesample      TTGGAGATTATGGGGCTGTGCGACAAGCGCTCGAGTGCTCTCCTTTTCGGTGACACTCGG
AB175624.1          TTGGAGATTATGGGGCTGTGCGACAAGCGCTCGAGTGCTCTCCTTTTCGGTGACACTCGG
                    ************************************************************

woodmicesample      CGCCTTTGTGGGAAATCCGCGGTTGCCTCGGCAGGCTTCGGCCTCGCAGAGAGTGCTTCC
AB175624.1          CGCCTTTGTGGGAAATCCGCGGTTGCCTCGGCAGGCTTCGGCCTCGCAGAGAGTGCTTCC
                    ************************************************************

woodmicesample      GTCTTCCCTCAACTCGCGGCATCCAGGAATGAAGGAGGGTAGTTCGGGGGAGAACGTACT
AB175624.1          GTCTTCCCTCAACTCGCGGCATCCAGGAATGAAGGAGGGTAGTTCGGGGGAGAACGTACT
                    ************************************************************

woodmicesample      GGTGCGTCAGAGGTGAAATTCTTAGACCGCACCAAGACGAACTACAGCGAAGGCATTCTT
AB175624.1          GGTGCGTCAGAGGTGAAATTCTTAGACCGCACCAAGACGAACTACAGCGAAGGCATTCTT
                    ************************************************************

woodmicesample      CAAGGATACCTTCCTCAATCAAGAACCAAAGTGTGGGGATCGAAGATGATTAGAGACCAT
AB175624.1          CAAGGATACCTTCCTCAATCAAGAACCAAAGTGTGGGGATCGAAGATGATTAGAGACCAT
                    ************************************************************

woodmicesample      TGTAGTCCACACTGCAAACGATGACACCCATGAATTGGGGAGTTTTTGGTCGTAGGCGGG
AB175624.1          TGTAGTCCACACTGCAAACGATGACACCCATGAATTGGGGAGTTTTTGGTCGTAGGCGGG
                    ************************************************************

woodmicesample      GTCGGGTTCATCTCGCTCCTCGTCTCGCCAATGTATATCAATTTACGTGCATATTCTTTT
AB175624.1          GTCGGGTTCATCTCGCTCCTCGTCTCGCCAATGTATATCAATTTACGTGCATATTCTTTT
                    ************************************************************

woodmicesample      TGGTCCTCGCAAGAGGTCCTCTTACGGGAAT-----------------------------
AB175624.1          TGGTCCTCGCAAGAGGTCCTCTTACGGGAATATCCTCAGCACGTTATCTGACTTCTTCAC
                    ****************************
```

Figure 3.4.Clustal W alignment of representative sequence of 18S rRNA gene detected in wood mice DNA (sample 67) with fragment of 18S rRNA gene from *T. grosi* species that was deposited in GenBank (accession number AB175624.1).

Table 3.3. The positive samples with highly similar sequences in the NCBI database

Rodent species	**Species of trypanosome**	**Number of positive samples**	**Identity**	**Accession number**
Bank vole	*Trypanosoma evotomys* 18S ribosomal RNA gene	1	100%	AY043356.1
Wood mice	*Trypanosoma grosi* 18S ribosomal RNA gene	3	100%	AB175624.1

The phylogenetic tree was constructed using two positive samples (representative samples) of the 18SrRNA gene in order to investigate the relationship between the positive sequences. The resulting Neighbor-Joining phylogram illustrated that the positive samples belonged to *Herpetosoma* species, *T. evotomys,* and *T.grosi* and that they were clustered with bank vole and wood mice samples, respectively (Figure 3.5).

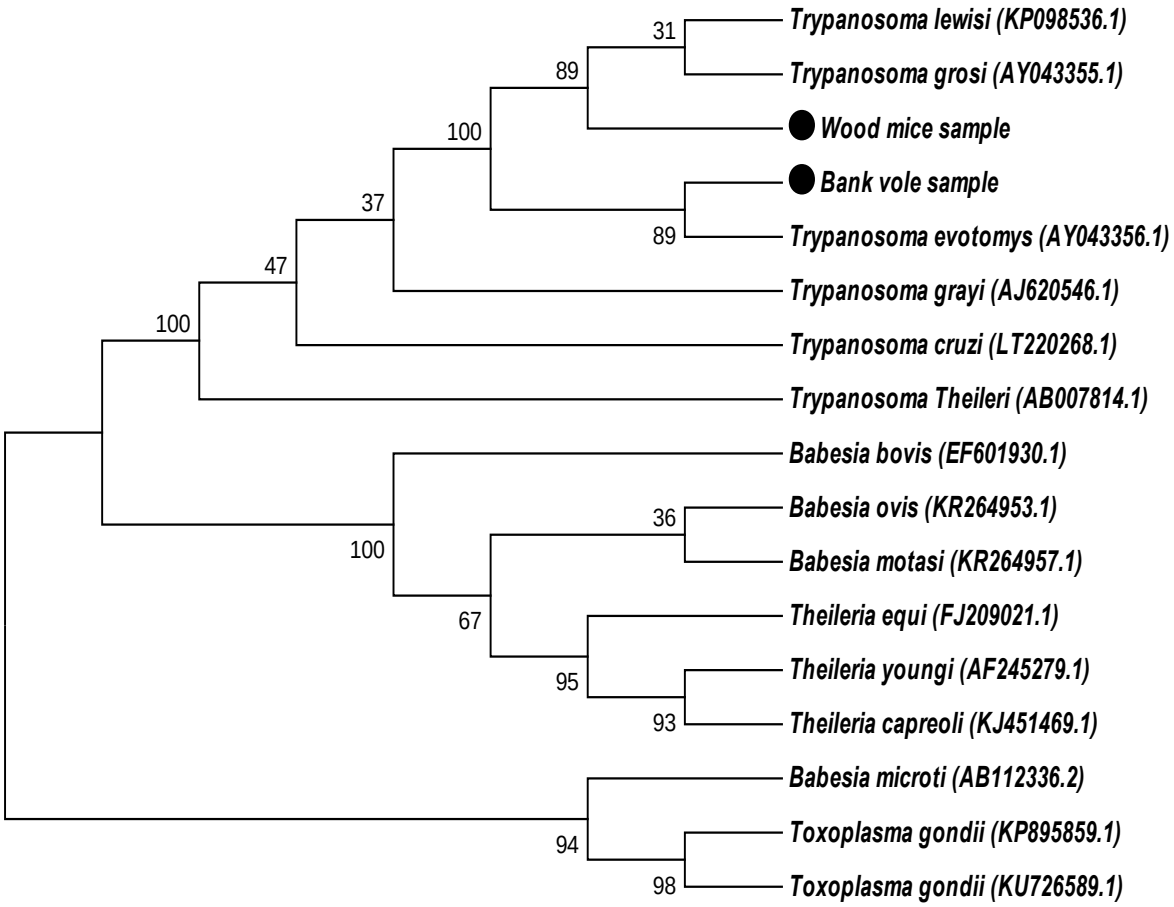


Figure 3.5. Phylogenetic tree for the *Trypanosoma Herpetosoma* using sequences analysis of 18S rRNA.

3.4. Discussion.

The investigation of different haemoparasites in small mammal species from England and Ireland showed that rodents were infected with both *Trypanosoma* species. In this study two species of the subgenus *Trypanosoma* (*Herpetosoma)* parasite were detected in bank voles and wood mice from the UK and Ireland, respectively. The overall prevalence of *Trypanosoma* spp in this study was 0.8% (4/473). In England, the prevalence of *Trypanosoma* infection by *T. evotomys* in bank vole was 1 out of 5 (20%) whereas 3 out of 179 (1%) of Irish samples (wood mice) were infected with *T. grosi*. The results demonstrated that the rodents were infected with the subgenus *Herpetosoma*. These results support the finding of Noyes and colleagues , who were able to detect *Herpetosoma* species from rodent blood (Noyes *et al.*, 2002). Interestingly, none of the wood mice that were collected from England were infected as well as the bank vole from Ireland. Furthermore, the detection of *Trypanosoma* DNA in shrew samples were not confirmed in this study. However, *Trypanosoma* described from other species of shrew such as *Sorex araneus* have been shown to be infected at a prevalence of 11% (9/79) from Northwest England, and the parasites had been suggested as possibly being an unknown *Trypanosoma* species since there was no published sequence data that was consistent with what was discovered (Bray *et al.*, 2007).

The observed prevalence of the infection in this study was lower than that previously reported in other studies in the UK (27%) (Noyes *et al.*, 2002). Two studies in Poland in 1996 and 2014 identified the prevalence of *T. evotomys* was 11% and 15.4%, respectively (Bajer *et al.*, 2014; Karbowiak & Sinski, 1996). The different observed prevalence rates of *Trypanosoma* spp in this study can be attributed to different factors such as the host being less exposed to infected vectors, a low level of parasitaemia in the blood, which could make detection of the parasite more difficult or it could be due to the fact that this was a different trapping season, where the vector and the parasites were less likely to be distributed in the environment.

Although, a high number of rodent species were collected from different sites in Ireland, *Trypanosoma* species was only detected in three wood mice in the site where no other species of rodents occurred in this area. In addition, no infection was reported in bank voles from other investigated areas, and it seems that those bank voles not infected with *Trypanosoma* species which present in Ireland. It is unclear why this is the case but there

are a number of possible explanations. Firstly, bank voles may be infected at a low level of prevalence in Ireland, so the absence of infection may be a consequence of sample size. Secondly, bank voles may not be susceptible to the native *Trypanosoma* spp due to the differences of the parasites or the host comparted to other researches. Furthermore, the bank vole could by innately resistant to *Trypanosoma* spp that are present in Ireland. However, many studies of rodent from European countries and other parts of the world were able to detect the parasites among bank vole populations (Bajer *et al.*, 2001). A study was conducted in rodent populations in Poland and the reported prevalence of *Trypanosoma* was 15.4% in bank voles (Bajer *et al.*, 2014). Thus, *Trypanosoma* species may be absent or at a very low prevalence in Irish bank voles. Investigation of other haemoparasites (*Bartonella* spp) in Irish rodents illustrated that similar results were observed where *Bartonella* DNA was only detected in wood mice at 50% (52/104), Indeed, no infection was reported from 93 bank voles, and it has been reported that the prevalence of *Bartonella* in wood mice declined significantly where both rodent species occurred. This can be attributed to the prevalence of the flea which increased in wood mice in areas without bank vole (Telfer *et al.*, 2005).

Studies by Bajer (2014) showed that the presence of the parasites had declined in older animals (Bajer *et al.*, 2014), which was also noted by Healing (1981) who suggested that the oldest animals might become resistant to this species due to acquired immunity(Healing, 1981). The alignment sequence of the bank vole and wood mice demonstrated that they had high similarity with *T. evotomys* (100%) and *T. grosi* (100%) respectively compared with other *Herpetosoma* species. Moreover, a longitudinal 18-month study by Turner (1986) was performed on bank voles and wood mice to assess different infections with several genera of haemoparasites such as *Trypanosoma*, *Babesia*, *Grahamella,* and *Hepatozoon*. Among each age group of the animal population, young animals were found to be infected with *Hepatozoon* and *Grahamella* while the other two parasites were found in older animals. Regarding seasonal changes, the bank vole was found to be more likely to be infected in summer and autumn, while no transmission was reported in winter and spring. However, a holistic examination of the study found that the true picture of infection in wood mice was not very clear due to the low number of the samples obtained from January to August 1982 (Turner, 1986).

Some studies regarding the phylogenetic relationship depends on the partial 18S rRNA sequences obtained from *Herpetosoma* species including *T. evotomys*, *T. grosi*, and

unknown *trypanosoma* species that were detected in wood mice (Noyes et al., 2002). Based on the phylogenetic tree of 18S rRNA, the results illustrated that all detected species from bank vole and wood mice in the UK and Ireland were closely related to *T. evotomys* and *T. grosi*. All detected species were clustered together with other species of *Herpetosoma* (Figure 3.5).

The presence of *Babesia* spp was not confirmed in this study. However, rodent and shrew species have been considered as reservoir hosts for different tick diseases such as *Babesia* spp. For example, study was conducted by Bown 2011 showed that *B. microti* was detected in shrew (*Sorex araneus*) with prevalence 30.3% (Bown *et al.*, 2011).Furthermore, *Babesia microti* was isolated from bank vole at 39% in Finland (Kallio *et al.*, 2014).

None of the collected rodents were positive for *Theileria* infection from rodent communities in this study. The only species of rodent known to be infected with *Theileria* species were dusky-footed woodrats (*Neotoma fuscipes*) (*T. youngi*) in northern California (Kjemtrup *et al.*, 2001). Our discrepant results could be attributed to the low level of parasitaemia in the blood or could be because the distribution of parasites was considerably less among the vector and reservoir hosts in the UK and Ireland.

Chapter Four

Haemoparasite infection of small mammals located in Saudi Arabia

4.1. Introduction.

Small mammals have been shown to be important hosts for a number of vector-borne infections. Most notably and recently, they appear to be critical in the putative re-emergence of plague in parts of the USA and Africa (Kosoy *et al.*, 2017; Nyirenda *et al.*, 2017) . A very recent publication has also demonstrated that there exists a wide distribution of endemic *B. microti* in Southwestern China, this need to do further investigations and monitoring of clinical disease in individuals presenting with Babesia like symptoms among those areas (Gao *et al.*, 2017). Small mammals like rodents can play important roles in the transmission of different zoonotic diseases such as *Toxoplasma gondii*, *Leishmania*, *Yersinia pestis* and *Bartonella* species (Inoue *et al.*, 2009; Kosoy *et al.*, 1997; Webster, 1994; Zhou *et al.*, 2004). Infection of rodent species by *Bartonella* has been investigated through a number of laboratory and field studies around the world using PCR. These studies illustrated that *Bartonella* are widely distributed in rodent communities (Birtles *et al.*, 1994; Kim *et al.*, 2005; Knap *et al.*, 2007; Kosoy *et al.*, 1997) . The first investigation of *Bartonella* infection in rodent species from Asia was performed in the Yunan province (South-Western China) (Ying *et al.*, 2002) . This study reported that *Rattus* rats were infected with *B. elizabethae,* which can infect many humans. Consequently, many studies have been conducted to detect the pathogen in rodent species in different tropical areas including Bangladesh, Indonesia, Thailand, and Vietnam (Bai *et al.*, 2007; Castle *et al.*, 2004; Winoto *et al.*, 2005).

To date, there is a little of information regarding the prevalence of *Bartonella*, *Babesia* /*Theileria* and *Trypanosoma* species in jirds (*Meriones libycus*) or other wildlife from Saudi Arabia, although there are reports of *Theileria* infection in cattle and sheep. Theileriosis was first reported in 1991 from a cattle population in Saudi Arabia (Al-Atiya *et al.*, 1991) and the disease is endemic in several Middle East countries. *T. annulata* was detected in cattle populations from different regions of Saudi Arabia such as Jazan and Riyadh, whereas *T. hirci* and *T. ovis* were detected in both species of sheep and goat (Al-Khalifa *et al.*, 2009). Information on other species of *Theileria* such as *T. youngi* has not been reported in the Middle East. The parasite was first detected in the dusky –footed woodrat (*Neomtoma fuscipes*) from northern California, USA (Kjemtrup *et al.*, 2001). In this study, amplification of 18srRNA illustrated that Libyan jirds and desert hedgehogs are considered to be important reservoirs for the pathogen.

Desert hedgehogs (*Paraechinus aethiopicus)* have been investigated for the presence of *Bartonella* and *Theileria/Babesia and Trypanosma* DNA, as it is known that hedgehogs can act as a host reservoir for a wide variety of pathogens including parasites, viruses, and bacteria, and these hosts can contribute significantly in the transmission of some zoonotic diseases (McCarthy & Moore, 2000). For example, transmission of *Salmonella* spp. associated with hedgehogs has been demonstrated (Woodward *et al.*, 1997). In the US State of Washington, an infant girl (10 months old) was infected with *Salmonella* type Tilene, which was believed to have derived from an African pygmy hedgehog raised by one member of the family (Control & Prevention, 1995) . In addition to this, *Cryptosporidium* spp. was considered to be the main cause of death in African hedgehogs (*Ateletrinx albiventris*) (Graczyk *et al.*, 1998).

Infection with *Bartonella* spp. and *Theileria* spp. has been reported in hedgehogs from a variety of countries (Bitam *et al.*, 2009). *Bartonella* spp. DNA was isolated from hedgehogs in Algeria, where the bacterium was detected in 12 of 75 (16%) trapped individuals. The DNA sequences of the 16S/23S genes indicated a high similarity to *B. elizabethae* (3/12) and *B. tribocorum* (9/12), while *Theileria* infection was detected in 21 out of 227 (9.2%) animals. Isolation of parasites from Chinese hedgehogs with similar identity to *T. luwenshuni* has been demonstrated, and this species has previously been shown to infect small ruminants (Chen *et al.*, 2014). Infection with that species has not been reported from desert hedgehogs in Saudi Arabia.

In Saudi Arabia, there is little information relating to tick borne disease amongst animal species, especially regarding the prevalence of *Theileria* infection in animal species such as cattle and sheep. However, the potential for investigation of tick borne disease has been shown where *Hyalomma spp* such as *Hyalomma anatolicum anatolicum* was shown to be responsible for the transmission of *Theileria* infection in Saudi Arabia between cattle and the sheep population (El-Azazy *et al.*, 2001; Hooshmand-Rad & Hawa, 1973). Therefore, there appears to be an evidential gap and this is a prime area of study which aimed to investigate the presence of *Bartonella* and/or *Theileria* species from jirds/hedgehogs in Saudi Arabia.

Aims

Libyan jirds and desert hedgehogs have been investigated for different haemoparasitic infections such as *Trypanosoma*, *Theileria*/*Babesia,* and *Bartonella*. The main aim of this chapter was to investigate the presence and characterise arthropod borne infection such as *Theileria/ Babesia, Bartonella and Trypanosoma* infection in Libyan jirds and desert hedgehog blood samples, collected from different parts of Saudi Arabia. The detailed objectives were as follows:

- Identify the prevalence of infection of different arthropod infection in Saudi wildlife.
- Identify different factor which may influence the probability of the host to be infected.
- Characterise the detected parasites and doing phylogenetic tree to compare them with other parasites which found in the world to explain the revolution history.

4.2. Methods.

The totall of 233 Saudi Arabian samples (121 gerbils and 112 hedgehogs) were collected by Dr. Abdulaziz (King Saud University, Riyadh, Saudi Arabia) from different areas of Saudi Arabia between 2014 and 2015. The blood samples were placed on flinders technology filter paper (FTA) card (Whatman) and sent to the University of Salford, UK for further study. All jird samples were of the Libyan jird type (*Meriones libycus*) and they were trapped in Riyadh. The total number of jird samples was 121 (61 male and 60 female). Data collection for the Libyan jirds 25/121 occurred in 2014 and 96/121 were trapped in 2015. The two species of hedgehogs, desert hedgehogs (*Paraechinus aethiopicus)* was collected from a wide variety of locations across Saudi Arabia whereas Brandt's (*Paraechinus hypomelas*) was collected from Albaha (Table 3.1). Demographic information such as gender (68/112 male, 44/112 female), age groups (92/112 were old, 20/122 were young), trapping location, and collection date were available. Once the samples were received at the University of Salford, The DNA was extracted according to the illustra™ Ready-To-Go™ GenomiPhi™ V3 DNA Amplification Kit protocol previously described in Chapter 2.2.3. This was done to augment sensitivity of the assays. After preparation of the DNA extract, DNA validity was confirmed by amplifying the mammalian tubulin gene. The presence of different parasites and bacteria was investigated

using a nested PCR, which uses specific primers for the aforementioned infections. Following the previous step, each PCR product was visualized using agarose gel electrophoresis (1.5%) in order obtain the correct band size, which could then be used to identify the presence of any parasites species. This study used specific band size for *Theileria* (600 bp) and for *Bartonella* (400 bp). The positive samples were purified as described in chapter 2.5. DNA concentration was measured according to the previous protocol described in chapter 2.6, and the samples were sent to the Source BioScience Company (Rochdale, UK) for sequencing. The obtained sequence was analysed using Finish TVa tool (http://officialsite.pp.ua/?p=2958497), and it was compared to the published sequences in genBank using the Blast tool. This study performed a calculation regarding the data prevalence of the infection for different analysis categories, such as gender, age groups, body condition, and seasonality in order to know which of those factors can play important role in maintaining and spreading the infection between animals. The proportion positive (prevalence) with 95% confidence intervals was used for all analysis categories. The prevalence of the infection was also compared between each analysis category. A phylogenetic tree was used to demonstrate the relationships between the species using the length (600 bp) and (331 bp) for *Theileria* and *Bartonella* sequences, respectively. This was conducted using MEGA6 software and branch support was assessed using 1000 replicates. The positives samples were compared with previously detected *Theileria* spp and *Bartonella* spp that were deposited in the Genbank. Further work has been conducted on *Bartonella* species by calculation of frequency of gltA sequence dissimilarity scores using a methods described by Pretorius et al (2004). This enabled the taxonomic relationship between bartonella detected in Jirds, hedgehog samples, and other closely related species to be examined. The 331 bp alignments contained 43 differences sequences; these included 5 representative jird samples (from 73 positive samples), 2 representative hedgehog samples (from 15 positive samples) and 36 species of *Bartonella* with valid published name. Pairwise comparison was performed among the 43 partial (331bp) gltA sequences. All the sequences were aligned together and the gltA sequence dissimilarities were calculated using MEGA 6. Pairwise comparison was determined between the 7 isolates (representative) and valid *Bartonella* species (36 species).

Table 4.1. Hedgehog species tested in this study from Saudi Arabia.

Regions	**Species**	**Number of samples**
Albaha	*Paraechinus hypomelas*	1
Badaya	*Paraechinus aethiopicus*	4
Buraidh	*Paraechinus aethiopicus*	4
Ghamas	*Paraechinus aethiopicus*	8
Majmaah	*Paraechinus aethiopicus*	3
Mlida airport	*Paraechinus aethiopicus*	29
Muznab	*Paraechinus aethiopicus*	2
Riyadh	*Paraechinus aethiopicus*	2
Riyadh Al khabra	*Paraechinus aethiopicus*	3
Shmasya	*Paraechinus aethiopicus*	1
Um Sedra	*Paraechinus aethiopicus*	1
Unizah	*Paraechinus aethiopicus*	54

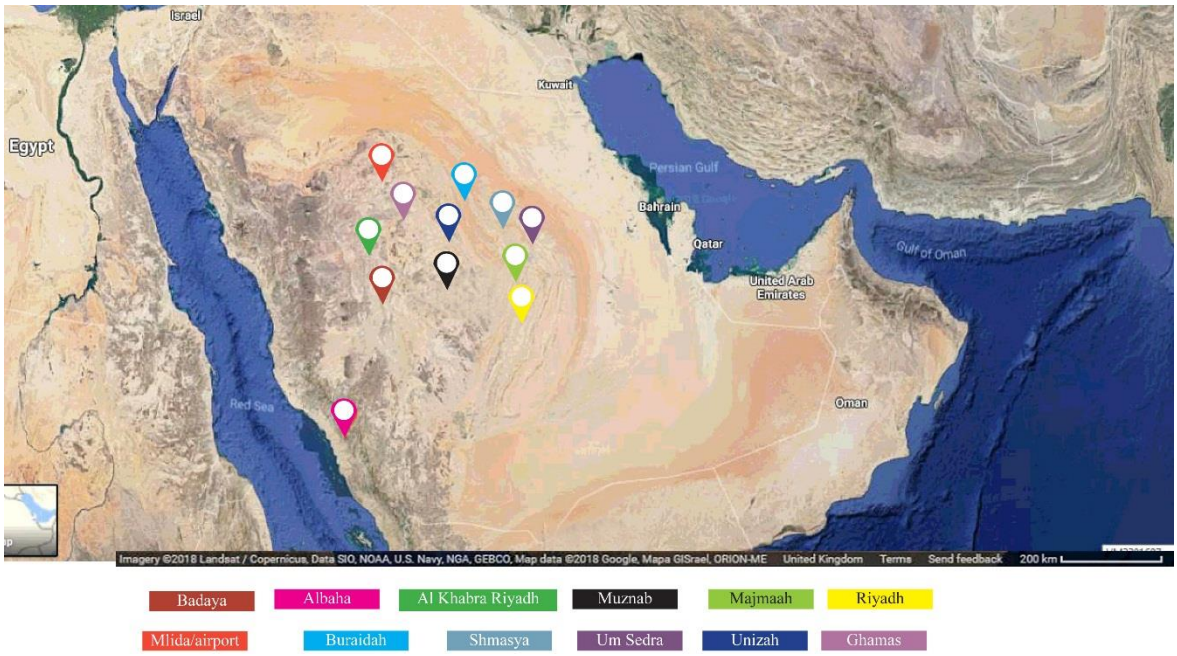


Figure 4.1. The collection locations for hedgehog samples from Saudi Arabia.

4. 3. Results

4.3.1. Mammalian tubulin gene identification

Tubulin gene was amplified (1000 bp) from all Libyan jird and desert hedgehog samples and the results illustrated that our samples were suitable for further assay (Figure 4.2).

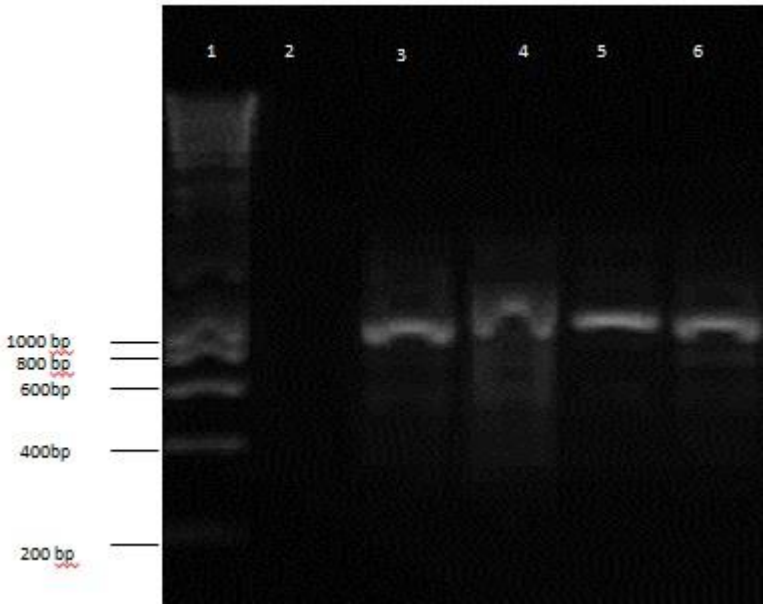


Figure 4.2. The PCR product for the tubulin gene is observed from tested samples using (1.5%) polyacrylamide gel electrophoresis. Lane: 1 represents the molecular weight marker (1kb ladder). Lane 2: negative control. Lanes 3 and 4: the loaded samples from Libyan jirds. Lanes 5 and 6: represent hedgehog samples. Bands on the picture indicate that DNA was successfully extracted.

4.3.2. Haemoparasitic infection in jird and hedgehog populations.

The jird and hedgehogs samples were investigated for different haemoparasitic infections including *Trypanosoma*, *Theileria*, *Babesia*, and *Bartonella* (Table 4.2).

Table 4.2. The positive samples for different haemoparasitic infections in jird and hedgehog samples.

Infection	**Positive Jird samples / total of jird samples**	**Percentage positive (Exact Binomial 95% confidence Intervals)**	**Positive hedgehogs samples / total of hedgehog samples**	**Percentage positive (Exact Binomial 95% confidence Intervals)**
Theileria spp	49/121	40 (31-49%)	74/112	66 (56-74%)
Bartonella spp	73/121	60 (51-69%)	15/112	13 (7-21%)
Trypanosoma spp	0/121	0 (0-3%)	0/112	0 (-3%)

From the above table it is clear that jird and hedgehog were infected by *Theileria* spp and *Bartonella* spp, and the presence of other haemoparasitic species such as *Babesia* and *Trypanosoma* spp were not confirmed in this study.

4.3.2. Prevalence of *Theileria* infection among Libyan jirds and desert hedgehogs.

The presence of *Theileria* spp was investigated using a nested PCR, and the overall prevalence of *Theileria* infection was shown to be 52% (123/233). The 123 (49/121) from jirds, (74/112) from hedgehog isolates in this study were identified as *Theileria* species based on DNA sequences of the appropriate 600 bp fragment of the 18SrRNA (Figure 4.3).

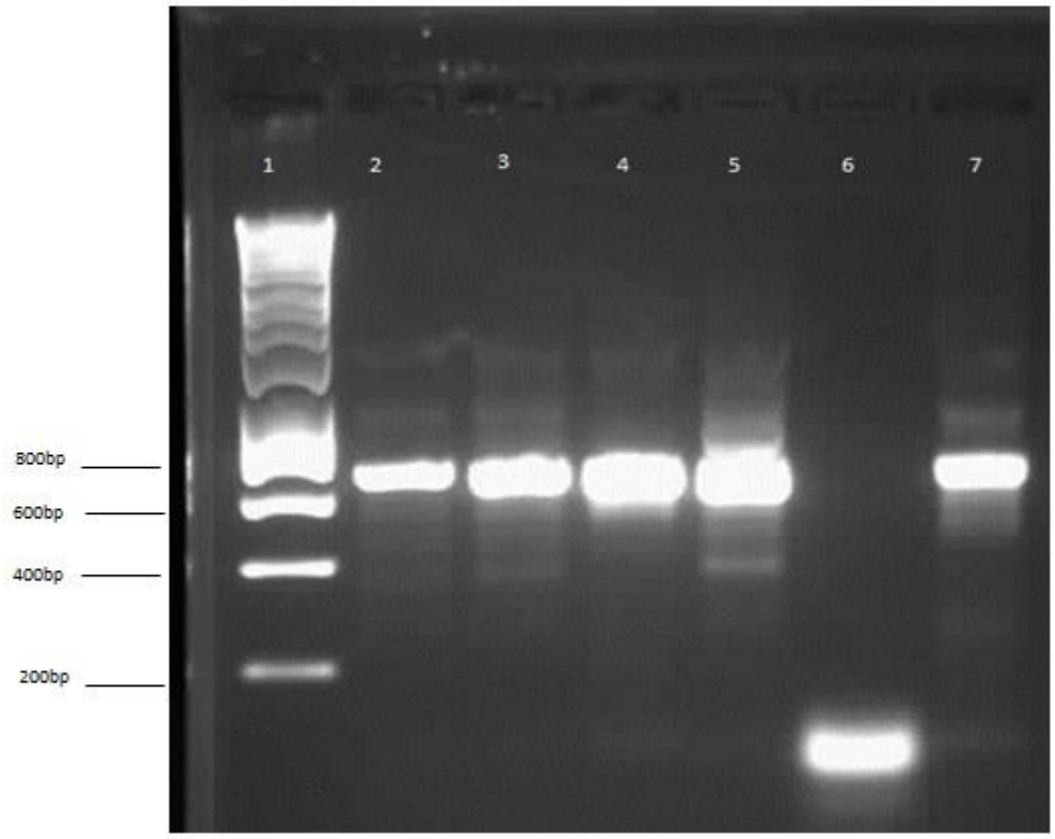


Figure 4.3. Gel image illustrates detection of *Theileria* spp in Jird and hedgehog samples. Lane 1: represents the molecular weight marker (1 kb). Lanes 2 and 3 are jird samples. Lanes 4 and 5: are loaded samples from hedgehog. Lane 6: negative control. Lane 7 is a positive control. Bands in lanes 2-5 indicate that samples were positive for *Theileria.*

Statistical analyses of gender showed that male and female jirds had a similar *Theileria* prevalence of 41% (95%CI 29-54%) (25/61) and 40% (95%CI 28-53%) (24/60) respectively, (Figure 4.4). There was no significant association between gender and probability of infection (χ^2=0.012, 1 d. f., P=0.91).

Figure 4.4. The prevalence of *Theileria* spp in male and female jirds from Saudi Arabia.

However, investigation of male hedgehogs demonstrated a prevalence of 73.5% (95%CI 61-83%) (50/68), which was significantly greater than the prevalence of *Theileria* in female hedgehogs (54.5% (95%CI 39-70%)) (24/44). Statistical analysis revealed that there was a significant difference between infected male and females with respect to infection with this parasite (χ^2=4.295, 1 d.f., P=0.038).

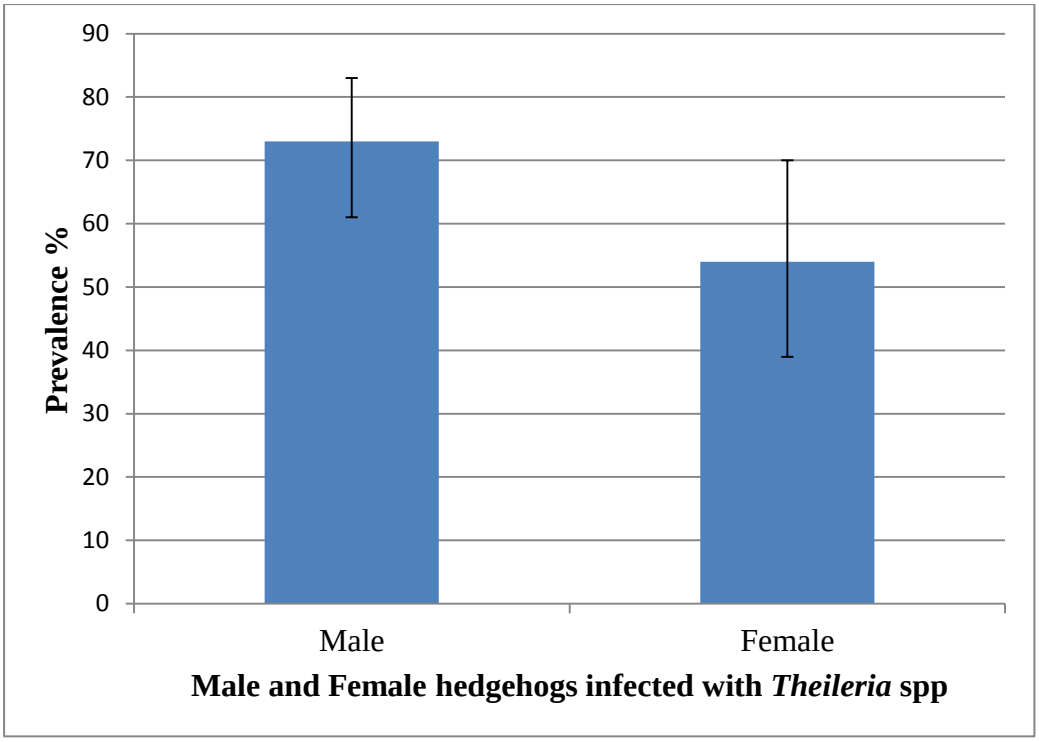


Figure 4.5. The prevalence of *Theileria* spp in male and female hedgehogs from Saudi Arabia.

The monthly incidence of *Theileria* spp in Libyan jirds (Table 4.3) and hedgehogs (Table 4.4) was analysed and we demonstrated that the prevalence of the infection in Jird samples was negligible (0%) in April to the maximum (73%) in July. We also recorded the rate of infection, which ranged from 11% to 54% during the months of February, March, October, and November, and from 55% to 73% in January, June, September, and December. However, there was no significant association between collection months ($P>0.05$) (Table 4.3).

Table 4.3. Monthly prevalence of *Theileria* species in Libyan jirds.

Month	**Number of tested samples**	**Positive samples**	***Theileria* prevalence (%) (95% CI)**
Oct-14	4	2	50 (6-93%)
Nov-14	11	3	27 (6-60%)
Dec-14	10	6	60 (26-87%)
Jan-15	11	6	55 (23-83%)
Feb-15	10	3	30 (6-65%)
Mar-15	10	2	20 (2-55%)
Apr-15	10	0	0(0-30%)
May-15	9	1	11 (0.2-48%)
Jun-15	9	6	67 (29-92%)
Jul-15	11	8	73(39-93%)
Aug-15	8	4	50 (15-84%)
Sep-15	10	7	70(34-93%)
Oct-15	5	1	20(0.5-71%)
Nov-15	3	0	0 (0-70.7%)

In hedgehog samples, the minimum prevalence of *Theileria* spp infection observed in hedgehog samples in this study was reported as 10% in August while the parasite was detected in 8 out of 8 (100%) samples in March. This was the highest prevalence recorded in this study(Table 4.4).

Table 4.4. Monthly prevalence of *Theileria* spp in hedgehogs.

Month	**Tested sample**	**Positive**	***Theileria* Prevalence (%) (95% CI)**
July 2014	10	7	70 (34-93%)
August 2014	10	1	10 (0.2-44%)
September 2014	9	4	44 (13-78%)
October 2014	13	7	53 (25-80%)
November 2014	8	3	37 (8-75%)
December 2014	1	1	100 (2-100%)
January 2015	10	9	90 (55-99%)
February 2015	10	6	60 (26-87%)
March 2015	8	8	100 (63-100%)
April 2015	10	7	70(34-93%)
May 2015	10	9	90 (55-99%)
June 2015	13	12	92 (63-99%)

The prevalence of infection in jird samples differed from 44% (95%CI 24-65%) (11/25) in 2014 to 39% (95%CI 29-50%) (38/96) in 2015. However, there was no significant difference between the collection years and probability of infection (P>0.05) (Figure 4.6).

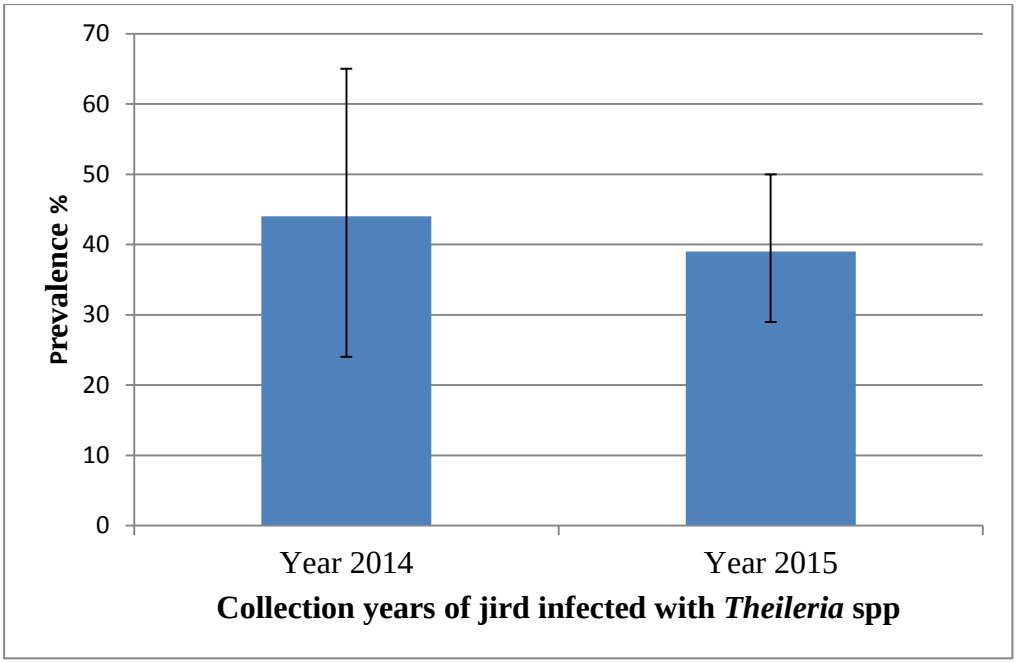


Figure 4.6. The prevalence of *Theileria* spp in Libyan jirds between 2014 and 2015.

Desert hedgehogs were reported to have a *Theileria* spp prevalence of 45% (95%CI 31-59%) (23/51) in 2014 which greatly increased to 83% (95%CI 72-91%) (51/61) in 2015 (Figure 4.7). This result illustrated a significant difference between the collection years and probability of infection in hedgehogs (χ^2= 18.375, 1 d.f., P=0.000).

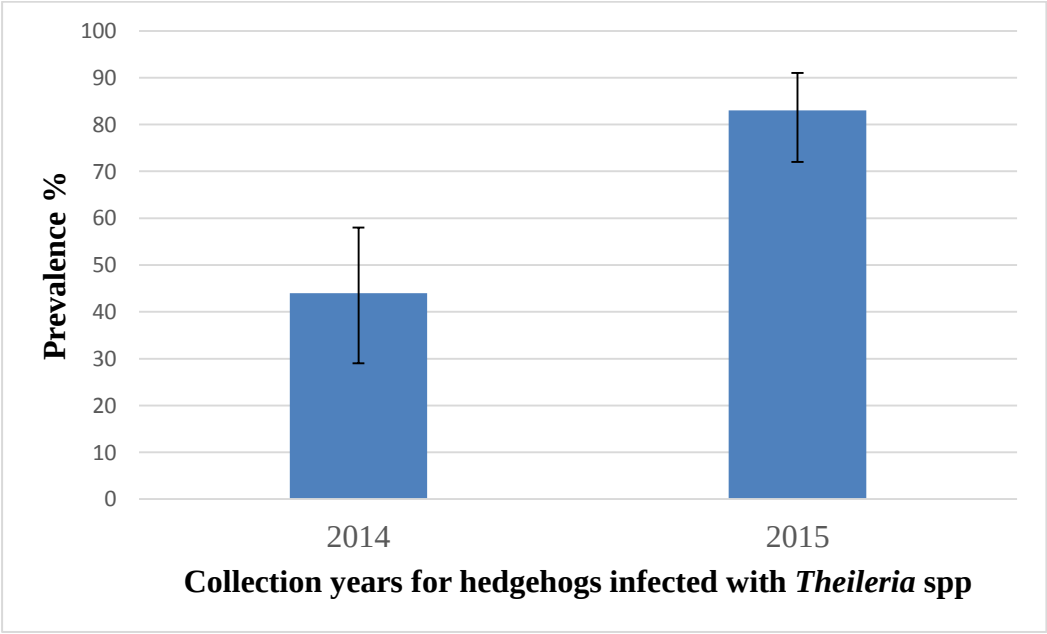


Figure 4.7. The prevalence of *Theileria* spp infection in dessert hedgehogs in 2014 and 2015.

For hedgehogs, data on the age of the individuals was provided by Dr Abdulaziz. The data show that infection with *Theileria* spp was detected in 65/92 (70.6%) (95%CI 60-79%) of old hedgehogs whereas it was detected in 9/20 (45%) (95%CI 23-68%) of younger animals, and this was a statistically significant difference, where age was associated with the probability of the infection (χ^2=4.822, 1 d.f., P=0.028).

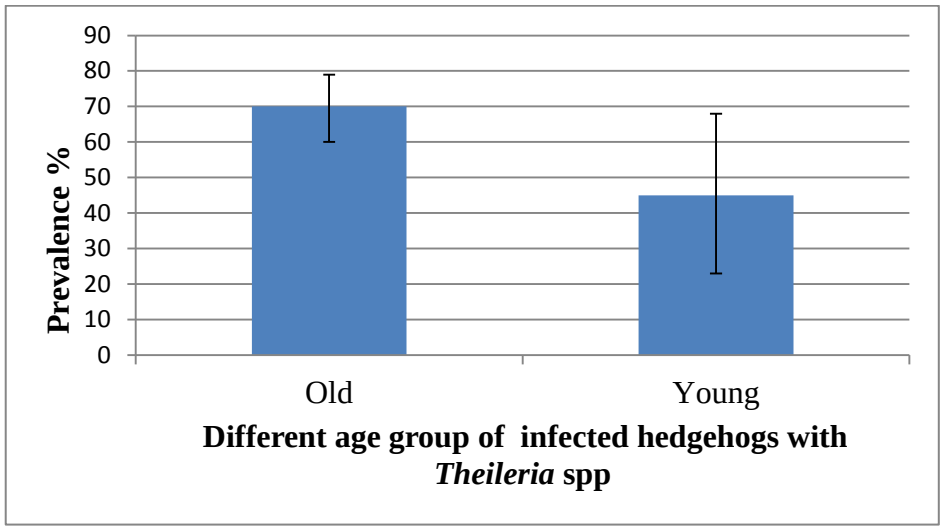


Figure 4.8. The prevalence of infected old and young hedgehogs with *Theileria* spp.

In addition to this, the hedgehogs were trapped from different regions (Fighure 4.9) in Saudi Arabia and it appears that prevalence of *Theileria* spp (Figure 4.10). varies between regions from 0 to 100%. For example, a high prevalence was observed in Riyadh100%(2/2), Ghamas 100%(8/8) , Buradiah 100%(4/4)and Muzunab 100(2/2) flowed by Mlida airport 86 % (25/29), Riyah Alkhabra 66% (2/3) ,Unizah 53% (29/54) and Badaya 50% (2/4).Interestingly, no *Theileria* infection was detected in collected samples from the Albaha, Majmaah, Shmasya and UM Sedra regions, although collections

were done at different times of the year.

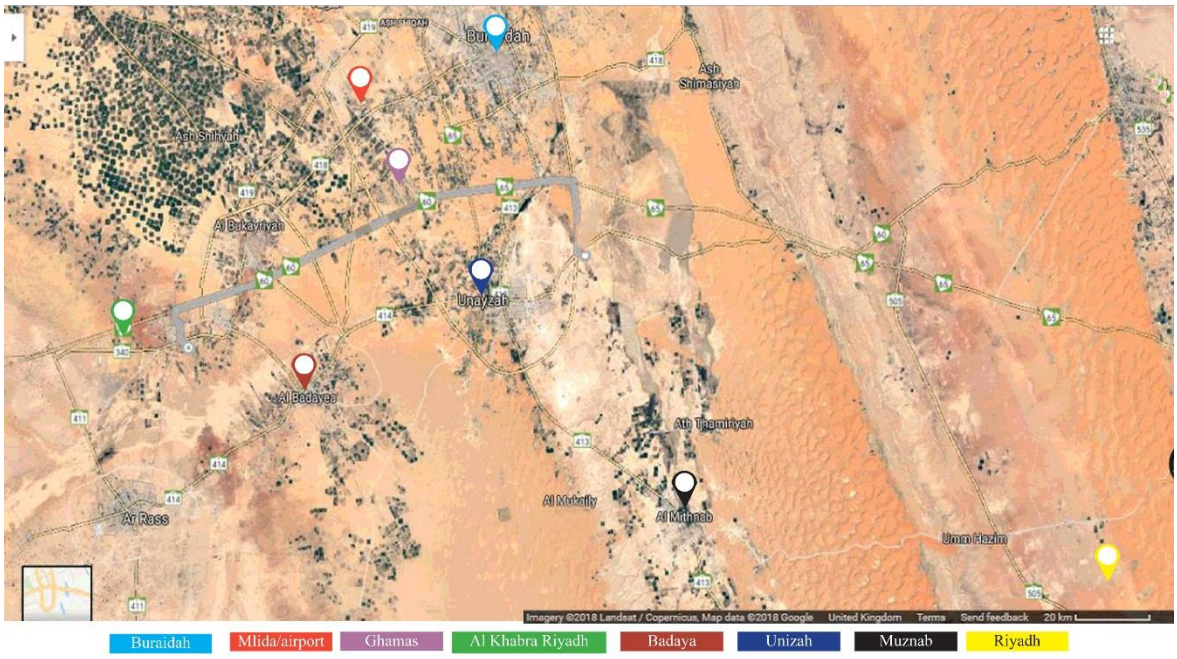


Figure 4.9. The collection locations for infected hedgehogs with *Theileria* spp.

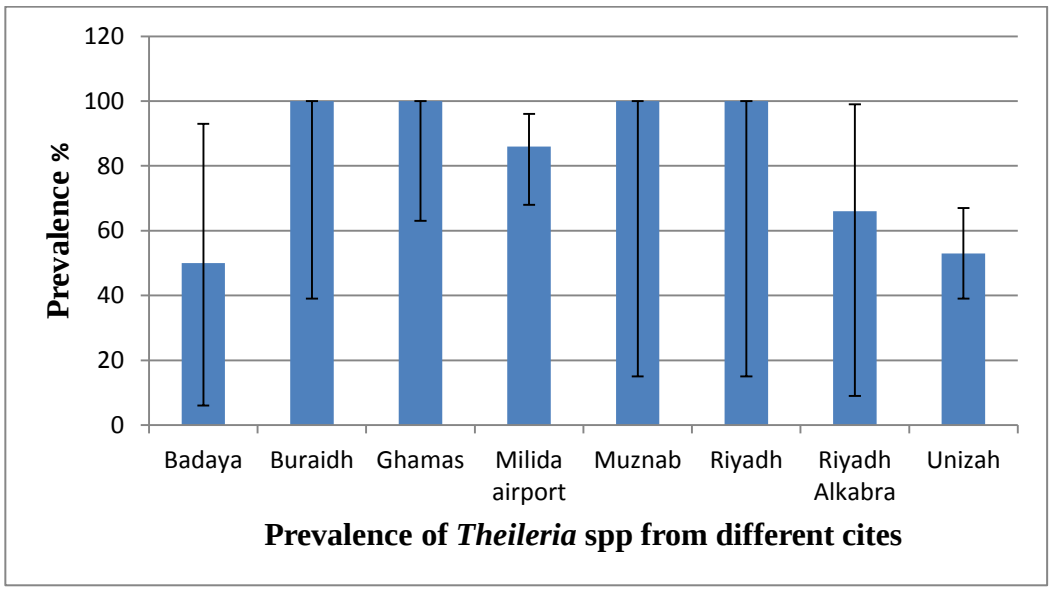


Figure 4.10. The prevalence of *Theileria* infection from different cities in Saudi Arabia.

Analyses indicated that for Libyan Jirds, no individual level factors (Sex, weight, coinfection) were significant predictors of the probability of an individual being infected *Theileria* spp. However for Desert Hedgehogs, heavier animals that were more likely to be infected (Coefficient = -0.00518, SE 0.00231; Chi-sq = 5.40, P=0.02), and males were

more likely to be infected than females (Coefficient = 0.860, SE 0.422; Chi-sq = 4.23, P=0.04).

4.3.3. Identification of *Theileria* species in Libyan jirds and desert hedgehogs from Saudi Arabia.

The obtained DNA sequences of parasites from infected jird and hedgehog samples were edited and excised appropriately to a final length of 600 bp. All infected samples in this study were shown to have 92% homology to *T.youngi* AF245279.1 (Table 4.5).

Table 4.5. All the sequences from the positives samples were similar to *Theileria youngi* (genbank accession no AF245279.1).

Species	Identity	Accession number
Theileria youngi 18S small subunit ribosomal RNA, complete sequence	92%	AF245279.1
Theileria capreoli clone 8P 18S ribosomal RNA gene, partial sequence	91	KJ451469.1

The constructed phylogenetic tree of neighbor-hood joining analysis describing the relationship of the 18S rRNA gene of *Theileria* species reported in this study and other species of *Theileria* deposited in GenBank is illustrated in Figure 4.11. All the positive sequences of *Theileria* species in this study were clustered with *T. youngi* (AF245279.1), which was originally reported from the dusky-footed wood rat from North California, USA.

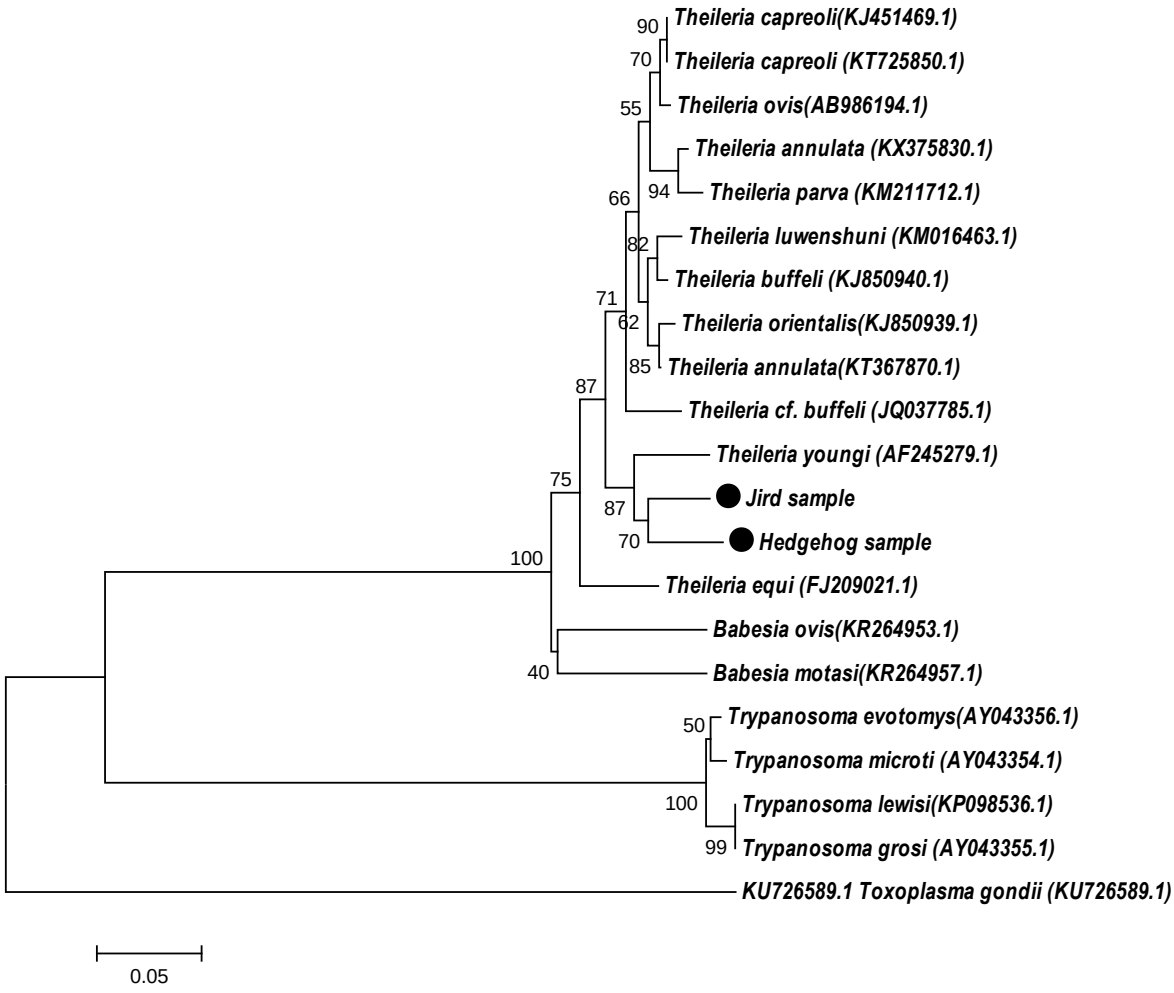


Figure 4.11. Phylogenetic analysis of the 18S rRNA gene constructed using Neighbor-joining test depicting the correct place of *Theileria* species detected in this study from infected jirds and hedgehogs.

4.3.4. Prevalence rate of *Bartonella* in jirds and hedgehogs from Saudi Arabia.

The citrate synthase gene (gltA) has been considered as a conserved gene, which permits detection of *Bartonella* DNA among different animal species (Figure 4.12).

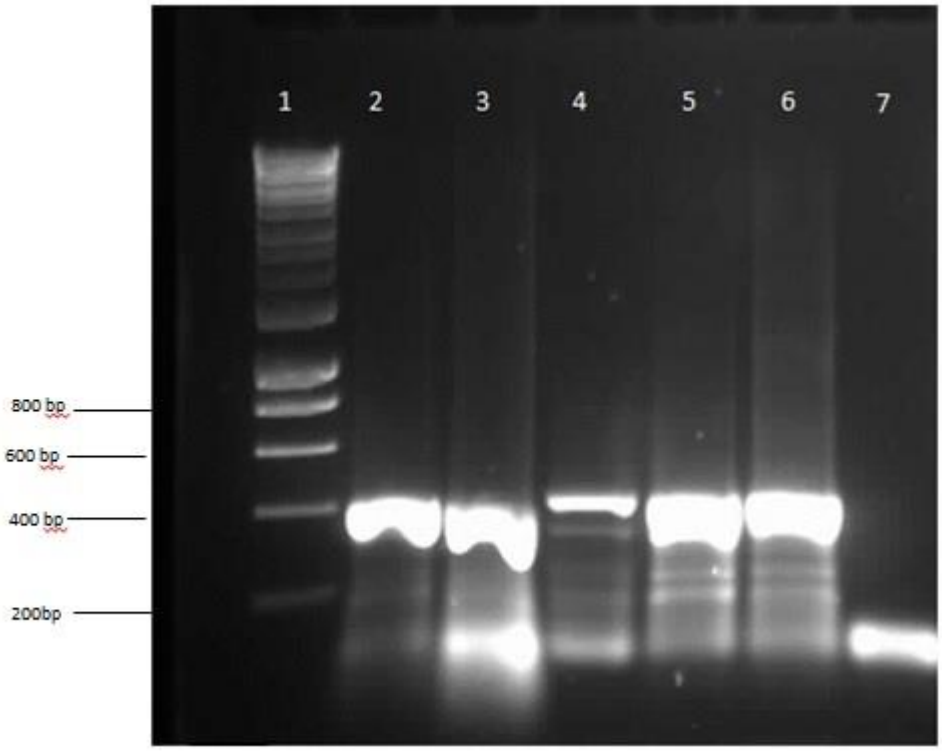


Figure 4.12. Gel image showing detection of *Bartonella* in jird and hedgehog samples. Lane 1: represents the marker (1kb). Lane: 2 and 3 are jird samples investigated for *Bartonella*. Lane 4 and 5 are loaded hedgehog samples and lane 6 is a positive control. Lane 7 is a negative control.

The successful amplification of the gltA gene resulted in identification of *Bartonella* DNA in 73/121 (37 male and 36 female) of the jird samples, while 15 samples were positive from hedgehogs (11 male and 4 female). Interestingly, male and female jirds had a similar prevalence rate of the infection at 60% with no significant difference reported between infected males and females (χ^2= 0.005, 1 d.f., P=0.9).

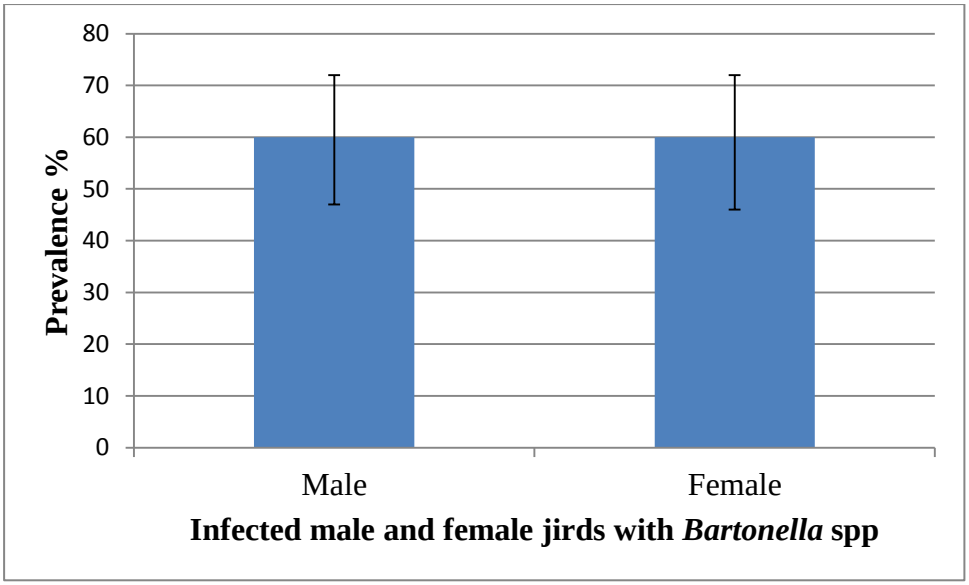


Figure 4.13. The prevalence of infected male and female jirds with *Bartonella*.

Our study of the hedgehog population showed that *Bartonella* was more likely to be detected in male hedgehogs at 16% (11/68 (95%CI 8-27%)) compared with females at 9% (4/44 (95%CI 2-21%)). However, the difference was not significantly different between infected males and females (χ^2=1.156, 1 d.f., P=0.28).

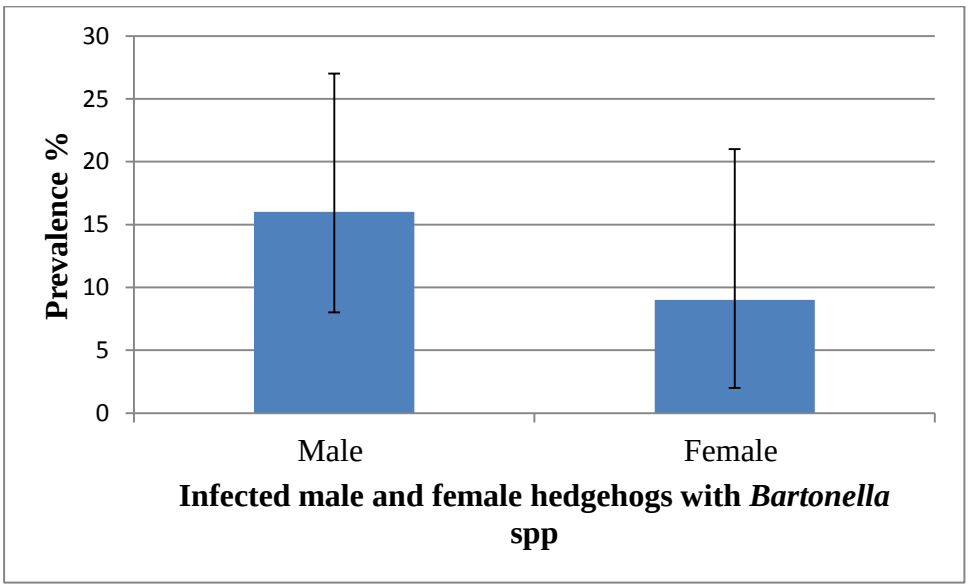


Figure 4.14.The prevalence of *Bartonella* in infected male and female hedgehogs.

The monthly prevalence of the infection was reported for jird (Table 4.6) and hedgehog samples (Table 4.7), and the results have shown that the prevalence rate of *Bartonella*

infection in jirds was reported between the minimum rate (20%) in February up to 100% in the months of June, July and October. The rate of the infection was reported to be between 26% to 60% during months of January, March, April, and December while it was also reported between 63% to 80 in May, August, September, and November (Table 4.6) However, there was no significant association between collection months (P>0.05).

Table 4.6. Prevalence of *Bartonella* spp. in jirds between October 2014 to November 2015.

Month	***Bartonella spp.***		
	Number of tested samples	**Positive**	**Prevalence**
Oct-14	4	1	25% (0.6-80%)
Nov-14	11	6	55% (23-83%)
Dec-14	10	3	30% (6-65%)
Jan-15	11	3	27% (6-60%)
Feb-15	10	2	20% (2-55%)
Mar-15	10	3	30%(6-65%)
Apr-15	10	5	50%(18-81%)
May-15	9	7	78% (39-97%)
Jun-15	9	9	100%(66-100)
Jul-15	11	11	100%(71-100%)
Aug-15	8	5	63%(24-91%)
Sep-15	10	8	80%(44-97%)
Oct-15	5	5	100%(47-100%)
Nov-15	3	2	67%(9-99%)

Bartonella DNA was detected in 15 samples. There was no infection in the samples that were collected in November 2014 and December 2014, whereas a high prevalence was recorded in July, August, April, and May at 20% (Table 4.7). However, there was no significant association between collection months (P>0.05).

Table 4.7. Monthly prevalence of *Bartonella species* in hedgehogs.

Month	Tested sample	Positive	Prevalence
July 2014	10	2	20% (2-55%)
August 2014	10	2	20% (2-55%)
September 2014	9	1	11 % (0.2-48%)
October 2014	13	1	7.6% (0.1-36%)
November 2014	8	0	0 (0-36%)
December 2014	1	0	0 (0-97%)
January 2015	10	1	10% (0.2-44%)
February 2015	10	1	10% (0.2-44%)
March 2015	8	1	12.5% (0.3-52)
April 2015	10	2	20% (2-55%)
May 2015	10	2	20% (2-55%)
June 2015	13	2	15.3% (1-45%)

DNA of *Bartonella* species was reported in 10 out of 25 (40%) (95%CI 21-61%) jirds in 2014 whereas a high prevalence of *Bartonella* infection was recorded in 2015 in 65% (63/96) (95%CI 55-75%) of tested samples. There was a significant association between collection years and probability of infection (χ^2= 5.442, 1 d.f., P=0.02).

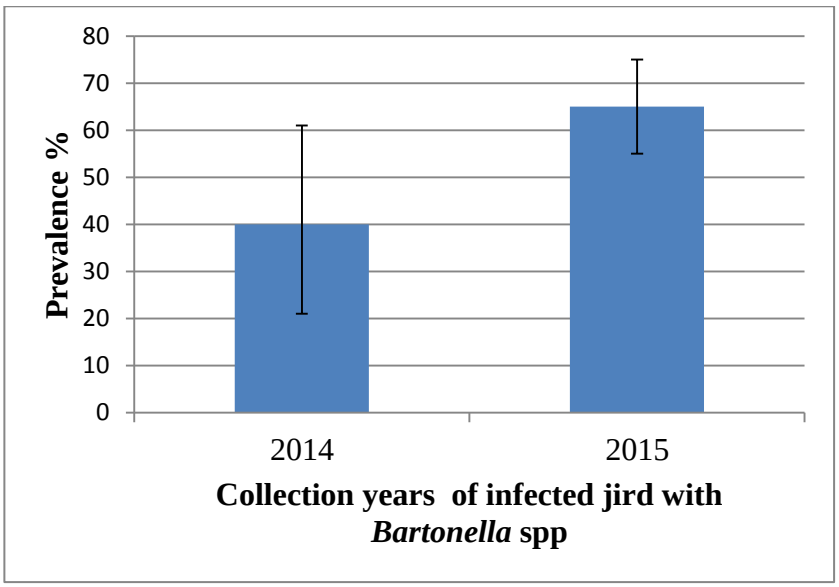


Figure 4.15. The prevalence rate of *Bartonella* infection in trapped jirds in 2014 and 2015.

In trapped hedgehogs, the prevalence of the infection was 12% (6/51 (95%CI 4-24%) in 2014 and14% (9/61 (95%CI 6-26%)) in 2015 (Figure 4.16). There was no significant association between collection years and probability of infection (χ^2= 0.151, 1d.f., P=0.69).

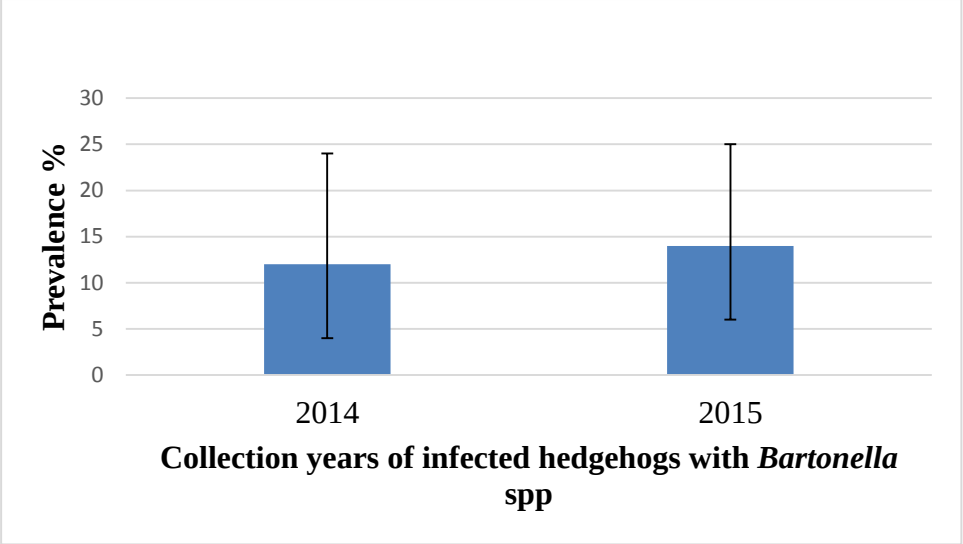


Figure 4.16. The prevalence rate of *Bartonella* infection in hedgehogs in 2014 and 2015.

In addition, we obtained information on different aged groups and this was provided by Dr. Abdulaziz. The results indicated the there was no significant differences regarding the prevalence of *Bartonella* infection between old (10.8%; 10/92) and young (25%; 5/20) hedgehogs (χ^2=2.828, 1d.f., P=0.093) (Figure 4.17).

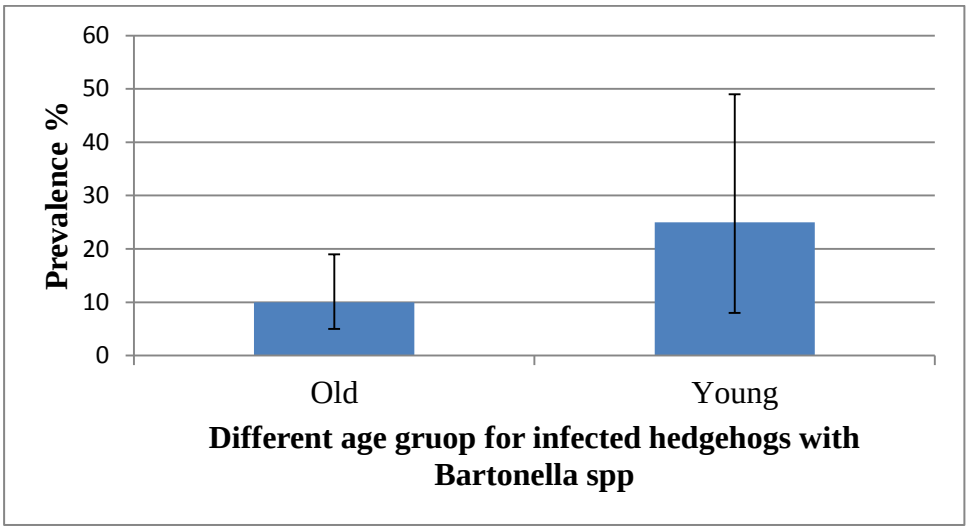


Figure 4.17. The prevalence of *Bartonella* in old and young hedgehogs.

This study assessed the prevalence of *Bartonella* parasites and how it differed for several regions in Saudi Arabia (Figure 4.18), with a range of 12% to 33% (Figure 4.19). Hedgehogs that were trapped in Riyadh Al Khabra were reported to have a high prevalence of the infection at 33% (1/3) flowed by Mlida airport at 20% (6/29),Ghamas at 12% (1/8) and Unizah at 12% (7/54). However, there was no significant association between collection areas(P>0.05).

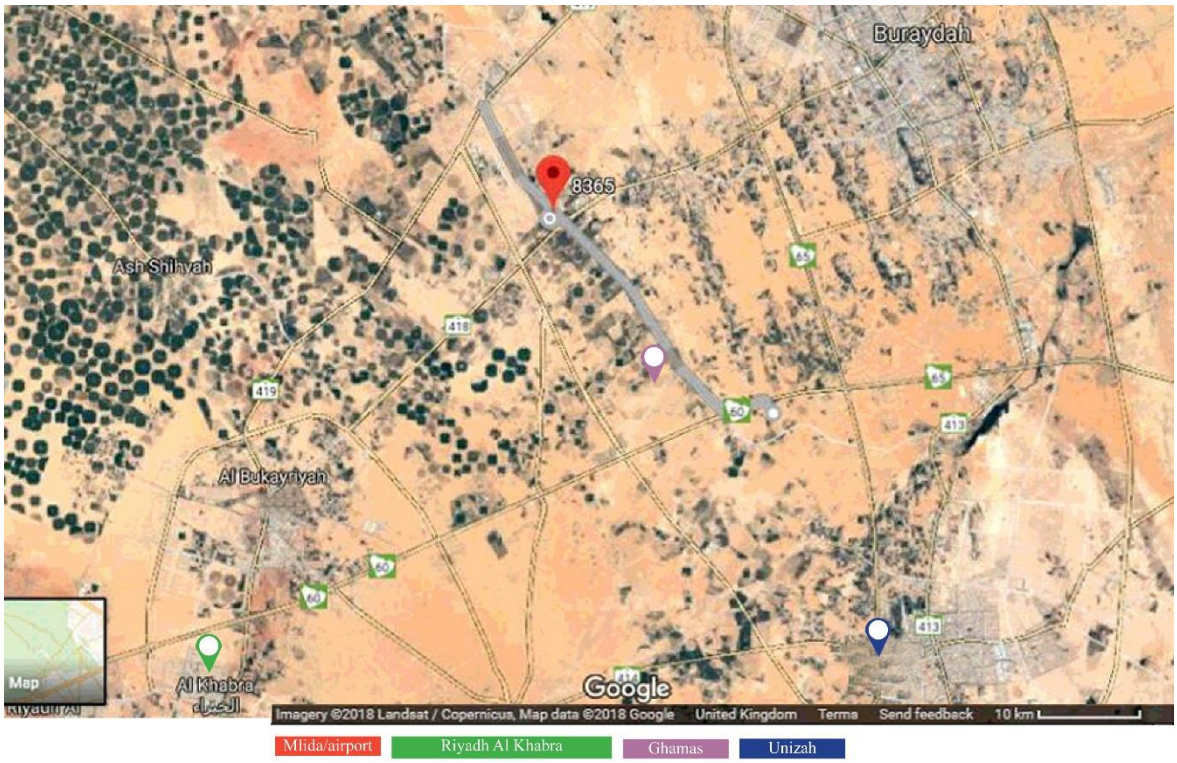


Figure 4.18. The collection locations for infected hedgehogs with *Bartonella* spp.

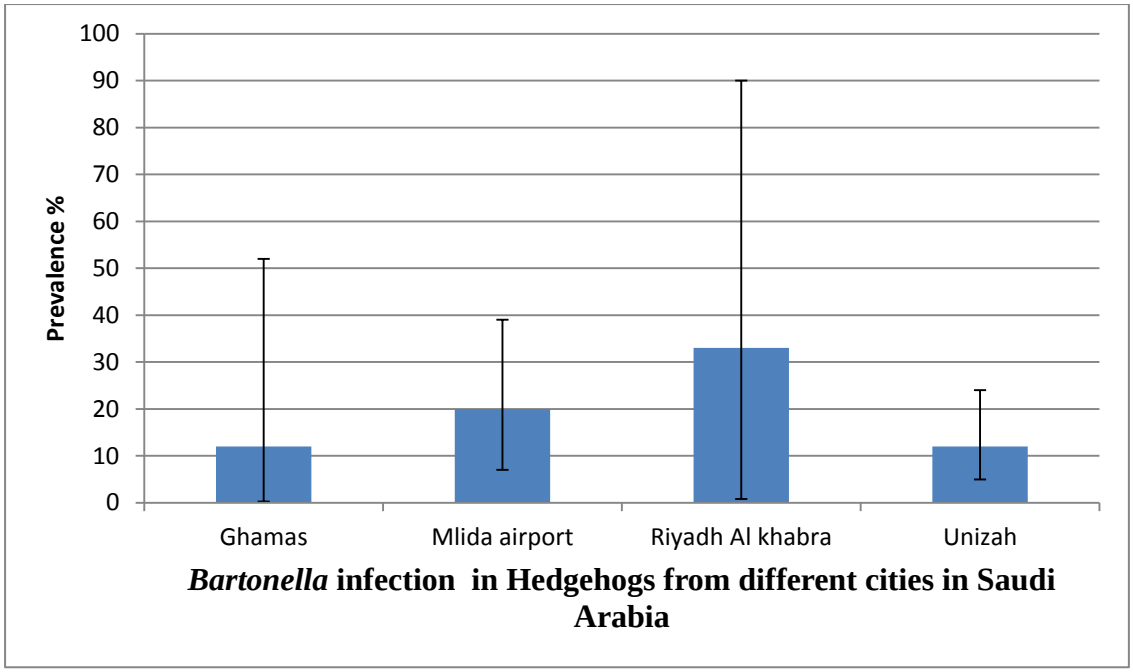


Figure 4. 19. The prevalence of *Bartonella* in hedgehogs from different cities in Saudi Arabia.

Analyses indicated that for Libyan Jirds, no individual level factors (Sex, weight, coinfection) were significant predictors of the probability of an individual being infected with *Bartonella* spp. However for Desert Hedgehogs lighter animals that were more likely to be infected (Coefficient = -0.00666, SE 0.00325; Chi-sq =4.59, P=0.032).

4.3.5. Identification of Bartonella species in Libyan jirds and desert hedgehogs from Saudi Arabia.

The comparison of partial citrate synthase gene (gltA) between animal species has demonstrated that 88 isolates (73 from jird and 15 from hedgehog samples) were infected by different *Bartonella* spp. Due to the large number of positives samples, we have used representative samples from both hosts for example: jird 1, jird 6, jird 10, jird 15, and jird 86 had a prevalence of 1.3% (1/73), 24.6% (18/73), 43% (32/73), 27.3% (20/73) and 3% (2/73) respectively. In comparison, hedgehog 79 and hedgehog 158 had a reported prevalence of 11/15 (73%) and 4/15 (27%) samples, respectively. The phylogenetic tree was constructed from alignment of these representative isolates with other bartonella species, which have been previously recognized. The Saudi jird and hedgehog isolates were formed of four clades from other *Bartonella* species.

The sequence analysis of partial gltA gene illustrated that none of our isolates were identical to other gltA sequences that were deposited in the GenBank. *B.elizabethae* (97% similarity) was prevalent among jird samples (jird 10) at a prevalence of 40% (32/73), while *B. grahamii* (92% similarity) was detected in two samples (3% prevalence (2/73)) , and one sample shared 98% similarity with *B. rochailime* at 1.3%. Four different isolates (representative samples were jird 6, jird15, hedgehog 97, and hedgehog 158) were clustered together (Figure 4.20). The similarity between the two isolates from the hedgehogs (hedgehog 158 and hedgehog 79) was 96%, which was the same proportion detected in jird samples (jird 15 and jird 5). Furthermore, it has been reported that hedgehog isolate (Hedgehog 158) shared similarity at 99% and 95% with jird isolate (Jird15) and isolate jird (Jirrd 6), respectively. The lower similarity evident among those isolates was reported between hedgehog isolate (Hedgehog 79) and jird isolate (Jird 6) at 94%. The most similar gltA sequence to those isolates (2 jirds and 2 hedgehogs) was *Bartonella pachyuromydis* with a maximum 8% dissimilarity.

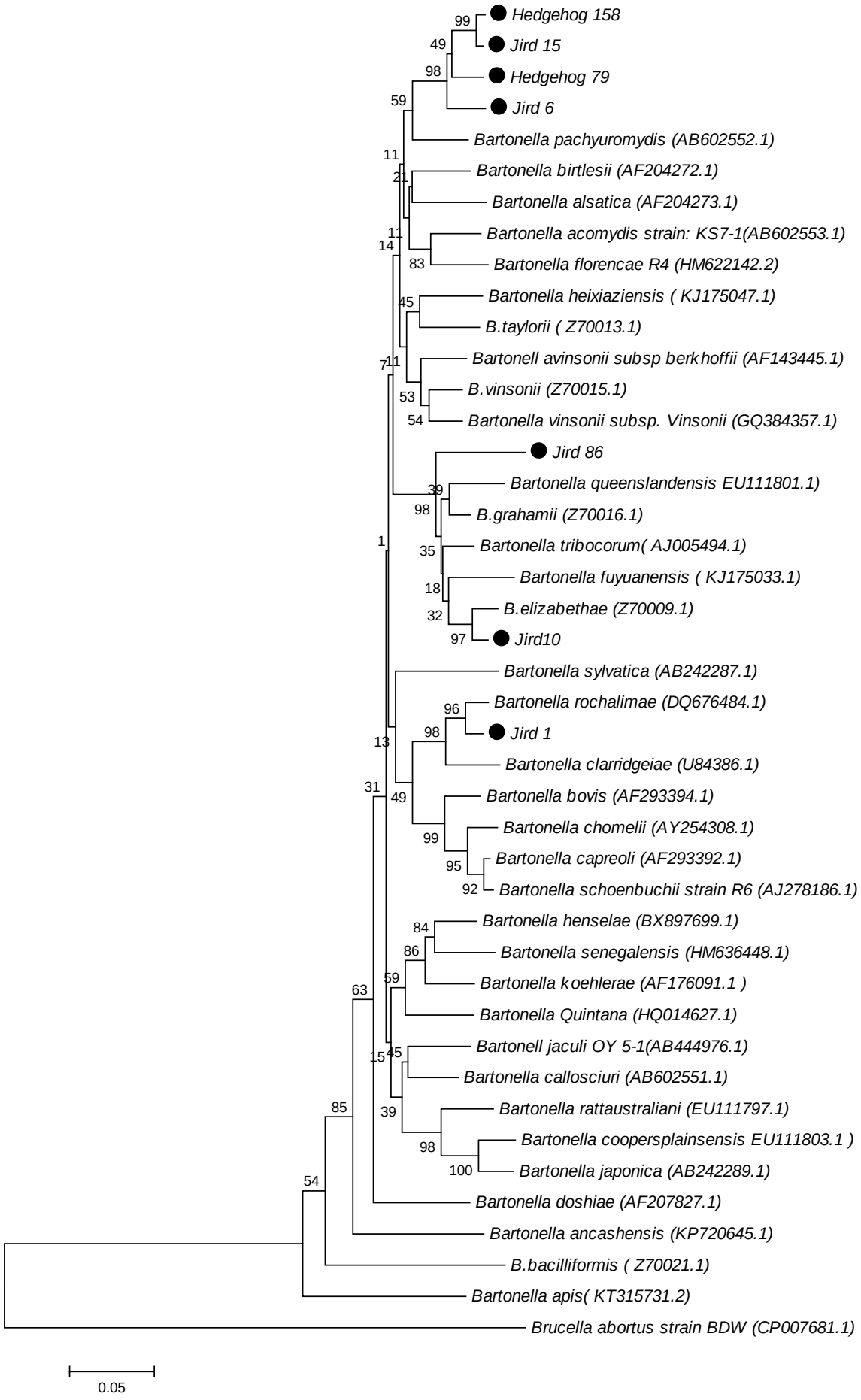
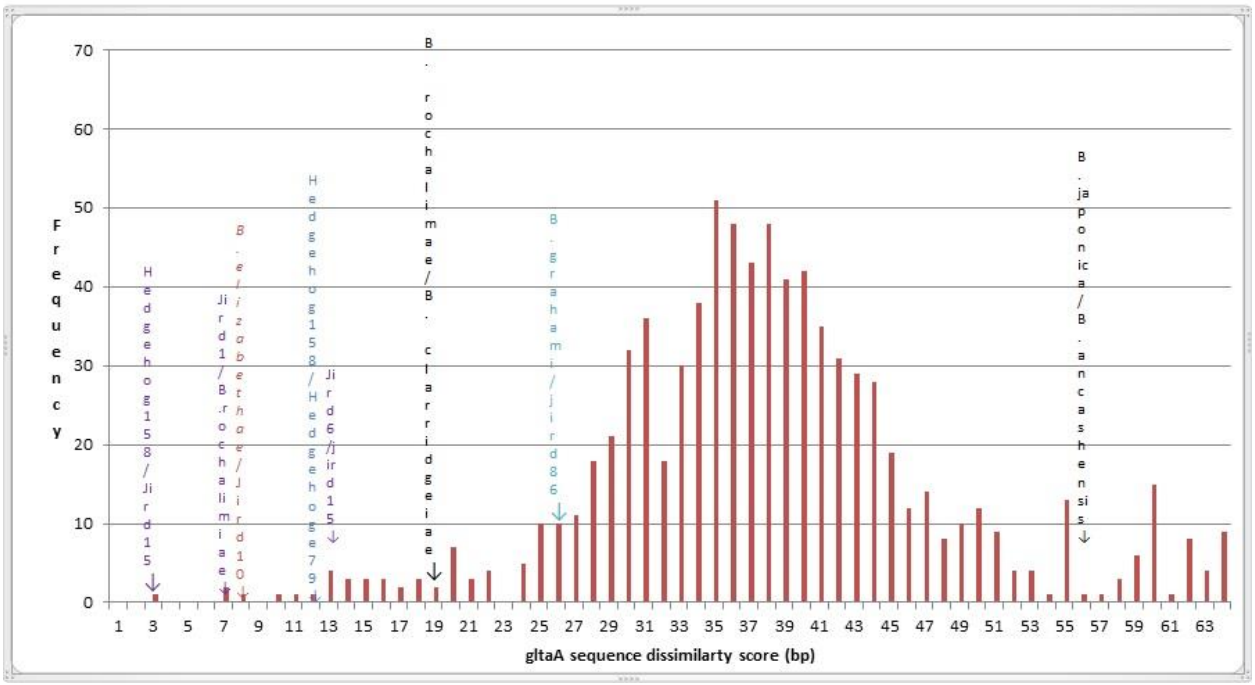

Hedgehog 158
Jird 15
Hedgehog 79
Jird 6
Bartonella pachyuromydis (AB602552.1)
Bartonella birtlesii (AF204272.1)
Bartonella alsatica (AF204273.1)
Bartonella acomydis strain: KS7-1(AB602553.1)
Bartonella florencae R4 (HM622142.2)
Bartonella heixiaziensis (KJ175047.1)
B.taylorii (Z70013.1)
Bartonell avinsonii subsp berkhoffii (AF143445.1)
B.vinsonii (Z70015.1)
Bartonella vinsonii subsp. Vinsonii (GQ384357.1)
Jird 86
Bartonella queenslandensis EU111801.1)
B.grahamii (Z70016.1)
Bartonella tribocorum(AJ005494.1)
Bartonella fuyuanensis (KJ175033.1)
B.elizabethae (Z70009.1)
Jird10
Bartonella sylvatica (AB242287.1)
Bartonella rochalimae (DQ676484.1)
Jird 1
Bartonella clarridgeiae (U84386.1)
Bartonella bovis (AF293394.1)
Bartonella chomelii (AY254308.1)
Bartonella capreoli (AF293392.1)
Bartonella schoenbuchii strain R6 (AJ278186.1)
Bartonella henselae (BX897699.1)
Bartonella senegalensis (HM636448.1)
Bartonella koehlerae (AF176091.1)
Bartonella Quintana (HQ014627.1)
Bartonell jaculi OY 5-1(AB444976.1)
Bartonella callosciuri (AB602551.1)
Bartonella rattaustraliani (EU111797.1)
Bartonella coopersplainsensis EU111803.1)
Bartonella japonica (AB242289.1)
Bartonella doshiae (AF207827.1)
Bartonella ancashensis (KP720645.1)
B.bacilliformis (Z70021.1)
Bartonella apis(KT315731.2)
Brucella abortus strain BDW (CP007681.1)
0.05

Figure 4.20. Phylogenetic analysis of the citrate synthase (gltA) gene was constructed using the Neighbor-joining test depicting the correct place for different species of *Bartonella* that were detected in this study from infected jirds and hedgehogs

The constructed phylogenetic tree of neighbor-hood joining analysis described the relationship of the gltA gene of *Bartonella* species reported in this study and other species of *Bartonella* that were deposited in GenBank .

In this study, the dissimilarity scores ranged from 1 to 64 bp and the frequency of intersequence dissimilarity scores were calculated for those changes which suggest that these frequency changes may fall into a bimodal distribution of two groups/modes. The smaller mode had frequency changes from 1 to 13 bp, while the large mode had frequency changes between 14 to 64 bp (Figure 4.21).



Figue 4.21. Bimodal distribution of inter gltA sequence dissimilarity score for Saudi rodent and hedgehog associated *Bartonella* with other representative *Bartonella* species.

4.3.6. Co-infection.

In this study, the prevalence of infection with *Thieleria* and *Bartonella* in jird samples was reported as 30/121 with no evidence of interaction between the parasites (χ^2=0.027 1 d.f., P=0.868). Similar results were observed in hedgehogs who were found to have *Theileria* and *Bartonella* in 8/112 samples with no evidence of interaction observed between the parasites (χ^2=1.25 1 d.f., P=0.263).

4.4. Discussion.

Wildlife species such as rodents and hedgehogs can play important roles in maintaining and transmitting a variety of zoonotic pathogens into susceptible human recipients. They act as an important reservoir of infection and are not easily controlled. For example, rodent species have been reported to be reservoir hosts for several microorganisms such as *Trypanosoma*, *Leishmania* and *Bartonella* (Alsarraf *et al.*, 2017; Ashford, 1996; Castle *et al.*, 2004; Sarataphan *et al.*, 2007; Ying *et al.*, 2002). Moreover, several rodent species are recognised as reservoir hosts for more than ten species of *Bartonella* spp, some of which are considered to be zoonotic such as *B.elizabethae, B.vinsonii* subset *arupensis, B.grahamii, B.washoensis* and *B.birtlesii* (Daly *et al.*, 1993; Saisongkorh *et al.*, 2009).

This study reports the first detection of *Theileria* and *Bartonella* in small mammal species such as jirds and hedgehogs in Saudi Arabia. Among the animal samples, the prevalence of *Theileria* was higher in hedgehogs (64%) compared with jird samples (40%) whereas the prevalence of *Bartonella i*nfection was reported at 60% and 13% in jird and hedgehog samples, respectively. This compares with a recent prevalence study of rodents in Egypt, which demonstrated that 7% of rodents were infected with Bartonellaceae (Alsarraf *et al.*, 2017). Molecular and phylogenetic analyses led to the description of two species: Candidatus *Bartonella fadhilae* n. sp. and Candidatus *Bartonella sanaae* sp (Alsarraf *et al.*, 2017).

Analysis of 18S rRNA sequences obtained from jird and hedgehogs illustrated that both hosts in Saudi Arabia are capable of harboring a different assemblage of *Theileria* genotypes that have also been reported in the cattle population from Saudi Arabia. The data from our infected jird and hedgehogs showed that these had 92% homology to the *T. youngi* strain, which was previously found in the North Californian Dusky-footed woodrat (Kjemtrup *et al.*, 2001). The prevalence in the woodrat population was high (61%) and in the study by Kjemtrup and colleagues (2001), infection was unrelated to gender or age of the woodrats. The organism was also identified from invertebrate vectors such as *Ixodes*, thus there is potential for this organism to be transmitted to humans.

In this study, we were able to demonstrate that there were significant differences between *Theileria* infections in relation to intrinsic variables such as age, weight, seasonality, and gender in hedgehog reservoirs. In China, the prevalence of piroplasm infections of hedgehogs discovered that *Theileria* was identified in more than a third of animals (35%)

(Chen *et al.*, 2014). *T. luwenshuni* was the responsible pathogen and infection did not result in any obvious symptoms, which would suggest that these parasites can inhabit their hosts for many years. One particularly intriguing finding revealed by the phylogenetic analysis, was that the *Theileria* spp. detected was very similar genetically to a *Theileria* spp. isolated from a feverish hospitalized patient in another part of China. Thus, it would appear to be the case that these reservoirs could harbor parasites that may be of public health concern. Although we identified differences in prevalence by both gender and age, an older study of the California dusky-footed woodrat did not show any differences by age or gender (Kjemtrup *et al*, 2001). However, our study is the first to show that there are clear differences in prevalence by age, gender, weight and seasonality in this particular animal reservoir.

New species and strains of *Bartonella* have been frequently detected recently and different genetic characterizations can be obtained by amplification of several *Bartonellla* genes including: rpoB, gltA, ribC, 16S-23S, groEL and 16S, which can permit clearer distinction between those species (Harrus *et al.*, 2009). The citrate synthase gene (gltA) has been widely used to distinguish between *Bartonella* species (Scola, Zeaiter, Khamis, & Raoult, 2003).

Analyses of the citrate synthase gene (gltA) demonstrated that different species of *Bartonella such as B. elizabethae* (97% similarity), *B. grahamii* (92% similarity), *B. rochailime (98%* similarity), and *Bartonella pachyuromydis* (92% similarity) were reported in jird samples in Saudi Arabia. *Bartonella pachyuromydis* was the most dominant with the highest prevalence of 52%. The organisms identified in our study were 92% identical to *Bartonella pachyuromydis* reported from the Netherlands (AB602552), followed by *B. elizabethae, B. grahamii, and B. rochailime* with a prevalence of 43%, 3%, and 1.3%, respectively.

In our study, the prevalence results for *Bartonella* infection in jirds did not differ significantly with respect to sex since males had similar prevalence to females. Thus, gender of the infected animal was not considered a specific risk factor for infection in this study. These results were similar to the study of *Bartonella* species detected in *R. norvegicus* in the South of France (Gundi *et al.*, 2004). However, it should be noted that the study by Gundi 2004 was a very small prevalence study of only 66 animals.

Few studies have been performed regarding *Bartonella* infection in hedgehog species. In this study, although *Bartonella* species were more isolated from males than female animals, there were no significant differences associated with the infection and gender difference was not identified as a specific factor for the infection. As there is a distinct lack of information pertaining to gender analysis regarding *Bartonella* infection in hedgehogs, we considered other host species (rat) in order to compare our findings. Similar results were reported from infected rat species in Taiwan (Tsai *et al.*, 2010). In this study different age groups was not identified as an important factor for *Bartonella* infection in hedgehogs as young animals were just as likely to be infected as older animals. Similar findings were reported by Kosoy et al (2004) who showed that there were no significant differences in the proportion of infected cotton rats by *Bartonella* species with different age groups. However, these findings contradict those of an early study, which examined prevalence of *Bartonella grahami* in field voles within the UK (Telfer *et al.*, 2007) . Over the course of three years, Telfer and colleagues showed that infection probability of B. *doshiae* and BGA (a novel strain) were most common in older host voles, but *B. graham* and *B. taylorii* were most common in non-reproductive hosts. Such a disparity in infection by species exemplifies the species-specific mechanisms of infection amongst this parasite.

Studies of prevalence of *Bartonela* infection in rodent species from some Asian countries are very limited comparing with other studies which have been done around the world. For instance, the presence of *Bartonella* DNA was investigated in rodent population from several location in Israel using real time PCR. The results showed that black rat (*R.rattus*) were had prevalence at 24% (19/79) while *Bartonella* DNA was detected at 25 % (1/4) from Cairo spiny mice. The detection species of *Bartonella* in infected rats were closely related to *B.tribocorum* and *B.elizabethae*. Cairo spiny mice was infected by *B.elizabethae* (Morick, 2009).Furthermore, study by Harrus in 2009 from Israel illustrated that 10 out 62 (16%) black (*R.rattus*) were infected with *B.elizabethae* which was similar to the detection species from Bangladesh, this phenomenon can suggests existence of widespread *Bartonella* species in Asian countries. In Thailand *Bartonella* was isolated in 17 out 195(8.7%) rodent species include bandicoot rat (*Bandicota indica*), black rat (*R.rattus*) lesser rice-field rats (*R. losea*), Ryukyu mice (*Mus caroli*),Polynesian rats (*R. exulans*). The result demonstrated that those rodents were infected with species that close related to *B. grahamii* and *B. elizabethae* (Castle *et al.*, 2004).

Investigation of *Bartonella* infection in hedgehogs from Saudi Arabia has illustrated that all isolates (15 positive) were closely related to *Bartonella pachyuromydis* (92% similarity), which were also reported in some jird samples. Different species of *Bartonella* was reported by amplification of the ITS gene from three hedgehog samples from Israel showed that 1 out of 3 (33%) was positive for *Bartonella* infection, and the sequence was closely related to *Bartonella* strain JB-15 (Marciano *et al.*, 2016).

Our findings may be of health importance as we found a high proportion of jirds in Saudi Arabia were infected with *B.elizabethae, B. rochalimae,* and *B. grahamii*. The potential for humans acquiring the infection is not very clear but it should be noted that this species has been isolated from several febrile patients in Thailand, Indonesia and the United States, presumably through zoonotic infection (Eremeeva *et al.*, 2007; Kosoy, Bai, *et al.*, 2010; Oksi *et al.*, 2013; Winoto *et al.*, 2005). Consequently, our research warrants further investigation on other reported species of *Bartonella* in Saudi Arabia.

This study did not report any evidence for interactions between infections in either of the host species studied, with no associations between infections with *Theileria* spp and *Bartonella* spp, despite them both being parasites of erythrocytes. This is in contrast to previous studies that have shown a that rodents infected by *Bartonella* spp are less likely to be infected by *B. microti* (Telfer *et al.*, 2010), probably as a consequence of competition.

The presence of *Babesia* spp. and *Trypanosoma* spp in jird and hedgehog was not confirmed since the all samples were negative.

Babesia species including B. *motasi*, *B. bigemina* and *B. cabali* have been recorded in host species such as sheep, cattle and horses in Saudi Arabia (Al-Khalifa *et al.*, 2009; Osman, 2017). Furthermore, there were a few studies regarding *Babesia* infection among rodent species in Saudi Arabia, in Riyadh city the prevalence of the infection was 30.65% from several rodent species such as *Rattus rattus, Rattus norvegicus, Mus musculus, Acomys c.dimidiatus, Jaculus jaculus, Merioness crassus,* and *Gerbillus cheesmani*. It was reported that *Rattus rattus* had a higher prevalence rate (34.5%) than other species while a low prevalence rate (20%) was observed among *G. cheesmani*. No infection was recorded in *Mus musculus* (Morsy, Bahrawy, Al Dakhil, & Abdel, 1994).

Trypanosoma species such as *T. evansi* are commonly found among camel populations in Saudi Arabia where their vector is present (Al-Afaleq *et al.*, 2015) . Rodent trypanosomiasis has not been investigated in Saudi Arabia. However, other species of

Trypanosoma such as *T. lewisi* have been detected in *Rattus rattus* (11.5%) from Iran (Seifollahi *et al.*, 2016).

The absence of *Babesia* and *Trypanosoma* species among jird and hedgehog populations can be attributed to the low parasitaemia, which was undetectable by PCR or can be attributed to the low prevalence of infection in the environment. It is hard to be sure about the reason of the absence of *Babesia* and *Trypanosoma* spp since limited number of studies have been done in this certain area.

Chapter Five

Haemoparasite infection in red foxes indigenous to the United Kingdom.

5.1. Introduction.

Tick-borne diseases are recognised as an important public health concern in many developing and developed countries around the world. Many haemoparasites including *Anaplasma* spp, *Ehrlichia* spp, *Theileria* spp, and *Babesia* spp have been reported in domestic and wild animals (Bishop *et al.*, 2004; Cacciò *et al.*, 2000; Fourie *et al.*, 2013; Ogden *et al.*, 2003).

The red fox (*Vulpes vulpes*) has been considered as a reservoir host for a variety of infections and parasites including *Bartonella* spp., helminths and *Babesia* spp. (Criado-Fornelio *et al.*, 2003; Fiocchi *et al.*, 2016; Henn *et al.*, 2009). Canine babesiosis may be caused by different species of *Babesia,* which are classified as large or small *Babesia* species (Kuttler, 1988). The large group of *Babesia* includes *B.canis, B.rossi* and *B.vogeli* whereas *B. gibsoni, B. conradae* and *Babesia vulpes* species (also known as *Theileria annae*) are considered to be small *Babesia* species (Camacho *et al.*, 2003; Criado-Fornelio *et al.*, 2003; Zahler *et al.*, 2000). *B. canis* is the predominant species of *Babesia* that infects dogs in Europe, and large numbers of cases have been recorded from Poland (Adaszek & Winiarczyk, 2008), Spain (Criado-Fornelio *et al.*, 2003) and Germany (Gothe *et al.*, 1989). Indeed, *B. canis* and *Theileria capreoli* were identified from wolf carcasses in Croatia, although the evidence of hemolytic disease was inconsistent with babesiosis in other animals (Polkinghorne *et al.*, 2017). In addition to this, *B. vogeli* and *B. gibsoni* have been reported from new and old world continents (Solano-Gallego & Baneth, 2011).

More recently, a potentially novel species known as *Babesia vulpes* was reported in foxes and it has been postulated that it may also infect domestic dogs. This species is closely related to *B. microti* (Zahler *et al.*, 2000). The parasites were detected in dogs suffering from renal failure, thrombocytopaenia, and anaemia in North–Western Spain (Camacho *et al.*, 2001).Recently, the parasites have also been detected in dogs from different regions of Europe including North Western Spain (Camacho *et al.*, 2001; García, 2006) , Sweden

(Falkenö *et al.*, 2013), Croatia (Beck *et al.*, 2009) and North-Western Portugal (Simões *et al.*, 2011).

Globally, the parasite has been identified by several laboratory studies in different canine species such as red foxes and domestic dogs using PCR . Between 1997-1999, Griado-Fornilo (2003) demonstrated that *Babesia vulpes* DNA was detected in 5 out of 10 red foxes from central Spain. In addition, PCR amplification of the 18s rRNA illustrated that 81 out of 404 (approximately 20%) Hungarian red foxes were infected with *Babesia vulpes* (Farkas *et al.*, 2015). Indeed, Orkun and Karaer, 2017 recently identified *B. vulpes* and *B. rossi* in foxes in turkeys, which illuminates the role of these parasites in wild animals and how they may impact upon onward transmission into domestic dogs.

Bartley et al , 2016 reported that of 316 lung exudate samples screened using a semi-nested PCR in order to determine the prevalence of *Babesia* in the UK fox population; the results illustrated that 46/316 (14.6%) of the screened samples were positive with 100% identity to *B. vulpes.* A high prevalence of the infection (36.7%) was reported in the central region of England, whereas no infection was reported in Wales from the 12 tested samples, albeit this is a very small number of samples tested. An obvious limitation of this study is the relatively small numbers tested for two countries the size of England and Wales and further prevalence studies should ideally encompass several hundred samples where logistics and finances allow.

A study by Barandika (2016)illustrated the presence of *Babesia* DNA species from red fox and badgers in Spain, using real-time PCR that targeted the 18SrRNA gene. The results demonstrated the presence of *B. vulpes* infection in 45.8% of red foxes, and all sequences were 100% identical to *Babesia* sp. isolated from a Spanish dog (*Babesia vulpes* AF18800). Two novel *Babesia* species, sequence types A and B, were detected in 52.5% of the badger samples. Both types shared 96.6% similarity and they had 98% homology, with the best hit in GenBank. The *Babesia* isolate from the Spanish dog (AF188001) has been described from 36 badgers (Type A) while *Theileria* spNH2 (Fj645725) was reported in 7 badgers (Type B) (Barandika *et al.*, 2016). Furthermore, the phylogenetic analysis of *Babesia* species in badgers showed that both types (A and B) were clustered with *Babesia* species that infect carnivores.

A study by Najm et al (2014) showed that 1,953 ticks were investigated for the presence of *Babesia* infection from vertebrate hosts. These included *Ixodes hexagonus, I. ricinus,*

Dermacentor reticlatus, and *I. canisuga.* In total, 118 (6%) ticks were positive for different *Babesia* species. *B. vulpes* was found in 13 samples of *I. hexagonus*, 8 of *I. ricinus* and 19 in *I. canisuga*. Two *Babesia microti* isolates, 46t(Jq8886058) and hlj72 (Jq993429), have also been identified in 2 *I. ricinus*, 1 *I. hexagonus*, and 1 *I. canisuga* ticks, while 2 *I. ricinus* ticks were infected with the second isolate (9.2%) and *Babesia vantorum* was detected in 5 *I. ricinus* samples (7.6%) with all 65 samples being sequenced. While there is a little of studies examining risk of *Babesia* species transmission from native *Ixodes* species in Scotland, an older case report did find putative evidence of transmission of *B. capreoli* to a sheep host, most likely by *Ixodes* ticks feeding initially on deer species (Purnell *et al.*, 1981). There is significant limitation in the number of studies that investigate the presence of other haemoparasite infections such as *Trypanosma* spp, *Theileria* spp, and *Bartonella* spp among fox populations around the world. The detection of those infections were not confirmed in this study. However, a serological survey in North Carolina and Virginia was able to report *Trypanosoma cruzi* DNA in grey foxes (*Urocyon cinereoargenteus*) only (Rosypal *et al.*, 2010). Furthermore, the isolation of *Bartonella* species such as *B. rochalimae* was reported in red foxes from Spain (Gerrikagoitia *et al.*, 2012) and the bacteria were also detected in collected fleas from red foxes in Hungary (Sréter-Lancz *et al.*, 2006).

Aims

The aim of this chapter was to investigate the prevalence of vector-borne infections in blood samples from the British red fox (*Vulpes vulpes*) collected from the environs of Bristol in the south-west of England. The detailed objectives are set out below:

- To investigate the prevalence of *Babesia, Bartonella,* and *Trypanosoma* infection in red foxes using PCR as a diagnostic tool.
- Identify factors associated with increased risk of infection such as gender, age, body condition, and seasonality.

5.2. Methods.

5.2.1. Sample collection, DNA extraction, and bioinformatics analysis.

A total of 392 fox blood samples were collected from the environs of Bristol in the south-west of England by the University of Bristol fox group. All foxes were casualties of road traffic accidents and therefore may not be truly representative of the wild red fox population. Blood samples were taken from the hearts of the carcasses. Information regarding the gender, age and body condition of the foxes was also obtained, together with information on when the carcasses were collected. Male foxes were 248 whereas 144 were female. Age was grouped into 3 distinct categories: 5 juvenile (less than 1 year old), 275young (1-3 years of age) and 112 adult (over 3 years old). Body condition was categorized as 10 with poor condition, 34 with fair condition, and 348 have good condition, notwithstanding any physical trauma from vehicles. Univariate analyses of those factors affecting the prevalence of the infection was performed for individual level factors, such as sex , age groups, body condition, and population level (seasonality) using chi-square test of association through the Minitab 17 (Minitab, Inc, USA).

DNA extraction from each sample was performed using a protocol previously described in Chapter 2.2.2. After preparation of the DNA extracts, mammalian tubulin genes were checked by the PCR protocol previously described in chapter 2.3.1. The detection of parasitic infection was performed using a nested PCR assay previously described in chapter 2.3.2. This included two PCR runs with four sets of specific *Babesia* primers that amplified the Beta tubulin gene. Following the previous step, each of the PCR products were visualized using gel electrophoresis in order to obtain the correct band size (see chapter 2.4.), which could then be used to identify the presence of any *Babesia* species. The positive samples were purified as described in chapter 2.5. DNA concentration was measured (see chapter 2.6) and sent to source biosciences for sequencing. Once the sequences were received, they were visualized and analyzed using Finch TV. The National Centre for Biotechnology Information (NCBI) was used to determine the similarity between detected species and other species that were deposed in Genbank. All sequences were aligned together using clustal alignment. A phylogenetic tree was used to show the relationships between the species and was performed using the length (170 bp) of the sequence with MEGA6 software.

5.3. Results.

5.3.1. Mammalian tubulin gene amplification.

The tubulin gene was amplified (1000 bp) from some fox samples and the results illustrate that our samples were suitable for further assay (Figure 5. 1).

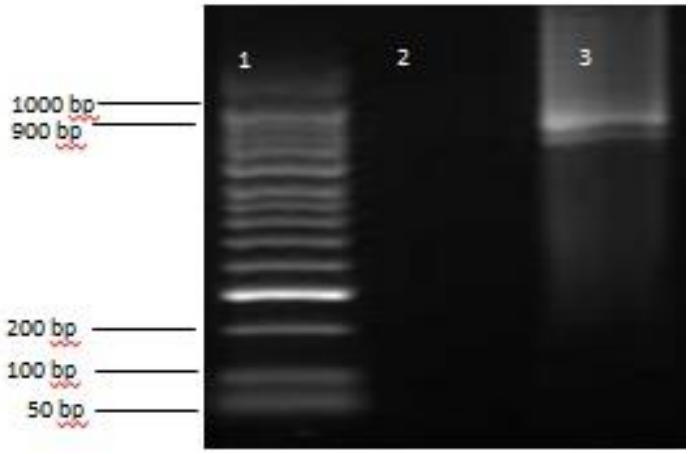


Figure 5.1. The Figure illustrates the PCR product with tubulin gene from fox samples using agarose (1.5%) gel electrophoresis. Lane 1 represents the molecular weight marker (50 bp). Lane 2 denotes the negative control. Lane 3 is the loaded sample. The band on lane 3 indicates that DNA was successfully extracted as this corresponds to the correct molecular weight.

5.3.2. Haemoparasite infections in the fox population.

The fox samples were investigated for different haemoparasite infections including *Trypanososma*, *Theileria*, *Babesia*, and *Bartonella* (Table 5.1)

Table 5.1. The positive samples for different haemoparasitic infections in red foxes.

Host	**Total samples**	***Typanososma* positive**	***Babesia* positive**	***Bartonella* positive**
Red fox	392	0	134 (34%, 95% CI 29-39%)	0

In this study, the only infection that was detected in fox samples was *Babesia vulpes.* The parasite was detected in 134/392 (34%, 95% CI 29-39%) fox samples and the corrected band size was obtained (Figure 5.2).

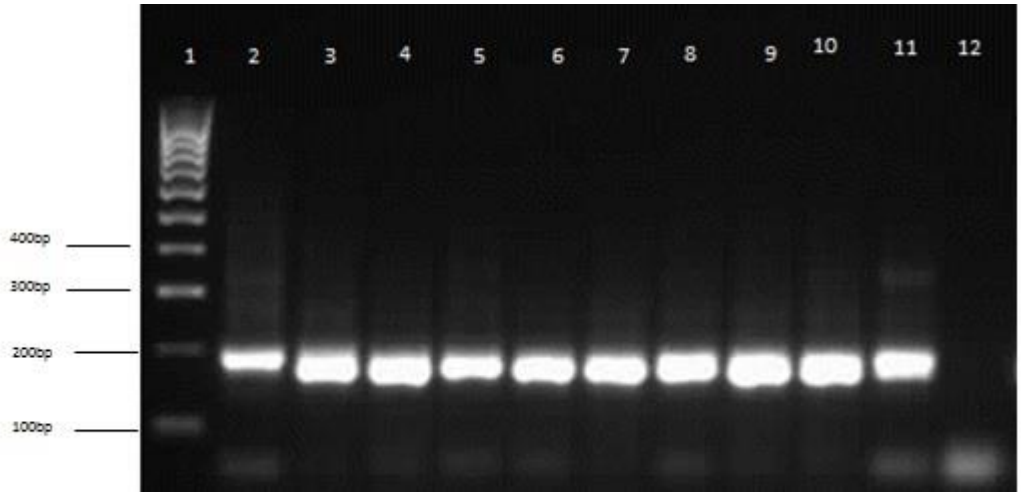


Figure 5.2. PCR amplicons of *Babesia vulpes* beta tubulin gene from fox samples by agarose (1.5%) gel electrophoresis. Lane 1 represents the molecular weight marker ladder (100 bp). Lanes 2 to 10 are samples investigated for *Babesia* using beta tubulin and nested PCR. Lane 11 is the positive control (6817 positive fox sample). Lane 12 is a negative control. Bands in lanes 2-10 indicate the samples were positive for *Babesia vulpes*.

The sequencing results illustrated the partial beta tubulin region of *Babesia vulpes* (Figure 5.3). The positive samples were identified as *Babesia vulpes* based on its band size (170 bp) and had 98% homology to *Babesia microti* isolate SN87-1 beta-tubulin gene (isolated from a fox in the USA) on the NCBI database, which confirmed its identity (Table 5. 2).

```
Foxsample       TAGGTTGGAACTTATGTAGGAGATAGTCCTTTTACATTAGAGAGAGCTGATGTTTTTTAC
AY144707.1      TAGGTTGGAACTTATGTAGGAGATAGTCCTTTACAATTAGAGAGAGCTGATGTTTTTTAC
                ********************************   ************************

Foxsample       AATCAATCAAGTGCTGGTAGATATGTACCAAGAGCCATTCTTATGGATTTAGAGCCTGGC
AY144707.1      AATCAATCAAGTGCTGGTAGATATGTACCAAGAGCCATTCTTATGGATTTAGAGCCTGGC
                ************************************************************

Foxsample       ACCATGGACTCCGTAA-------------------------------------------
AY144707.1      ACTATGGATTCTGTTAGATCAGGACCTTATGGTGAATTATTCCGTCCAGATAACTATGTA
                ** ***** ** ** *
```

Figure 5.3. Clustal W alignment of representative *Babesia vulpes* beta tubulin gene PCR products derived from fox sample, with fragments of the beta tubulin gene from *B. microti*-like species *(T. annae)* isolate SN87-1 (GenBank accession number AY144707).

Table 5.2. Blast summary data for *Babesia vulpes* beta tubulin gene PCR product.

Highly similar sequences	**Sample number**	**Query cover**	**Identity**	**Isolated host**	**Accession number**
Babesia microti isolate SN87-1 beta-tubulin gene	positive samples	92	98	fox	AY144707.1
Babesia microti isolate Rula nonfunctional beta-tubulin	positive samples	98	92	dog	AY144709.1

gene					

The phylogenetic tree was assumed using one positive sample of the beta tubulin gene in order to investigate the relationship between the positive sequences. The resulting Neighbor-Joining phylogram illustrated the positive samples belonged to *Babesia vulpes*, the samples were clustered with *Babesia microti*-like species that were isolated from fox samples (AY144707.1) (Figure 5.4).

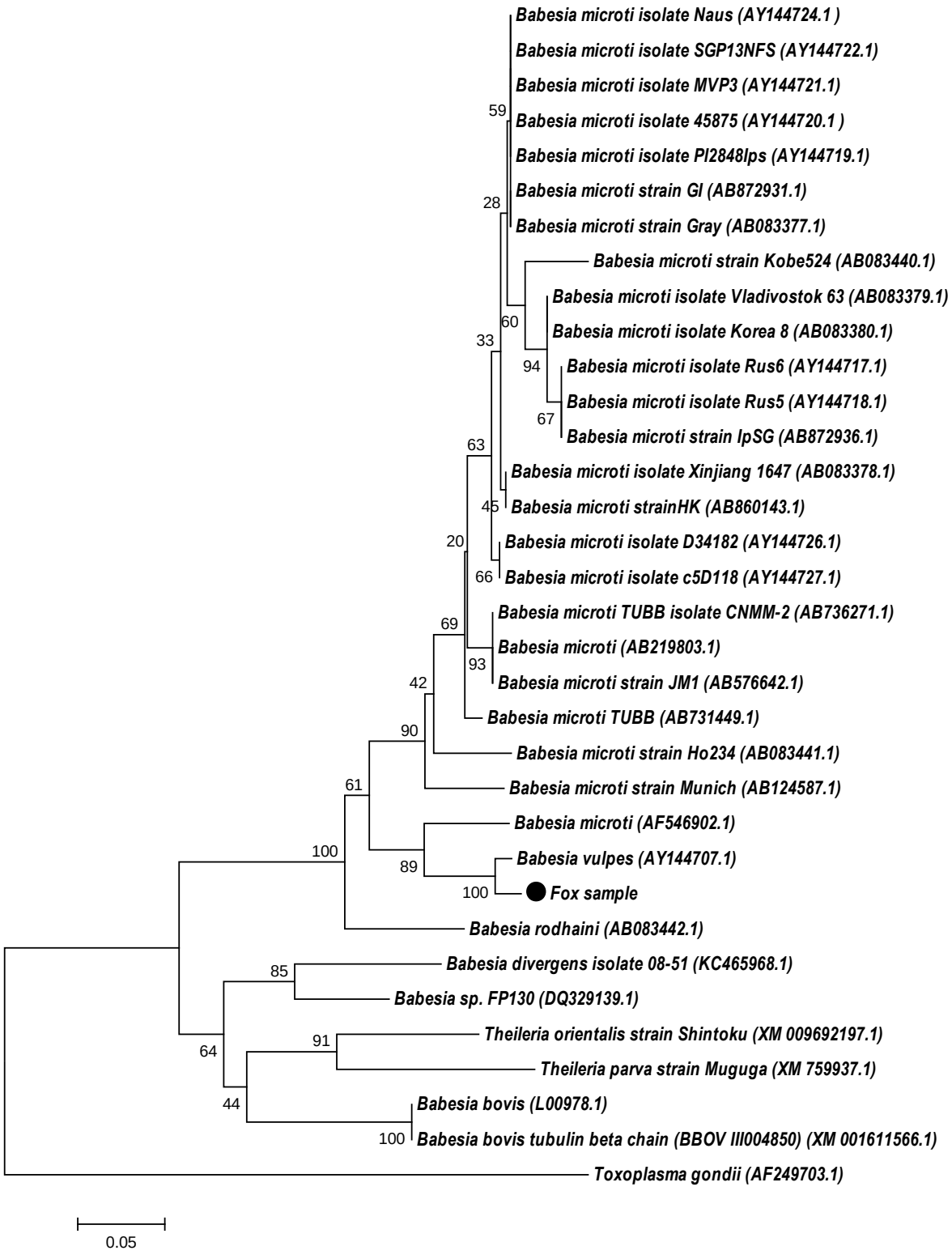


Figure 5.4. Phylogenetic analysis of the beta tubulin gene was constructed using the Neighbor-joining test depicting the correct genetic location of Babesia species detected in this study of infected red foxes (Brown circle).

5.3.2.1. Infection of *Babesia vulpes* based on sex.

The prevalence of the infection was reported as being 54/144 females (37.5%, 95% CI 29.9-45.6) and 80/248 in males (32%, 95% CI 26.7-38.3) (Figure 5.5). There was no significant association between sex and probability of infection (χ^2=1.113, 1 d.f., P = 0.292). This is most likely due to small numbers in the study and might attain significance should larger sample size be used.

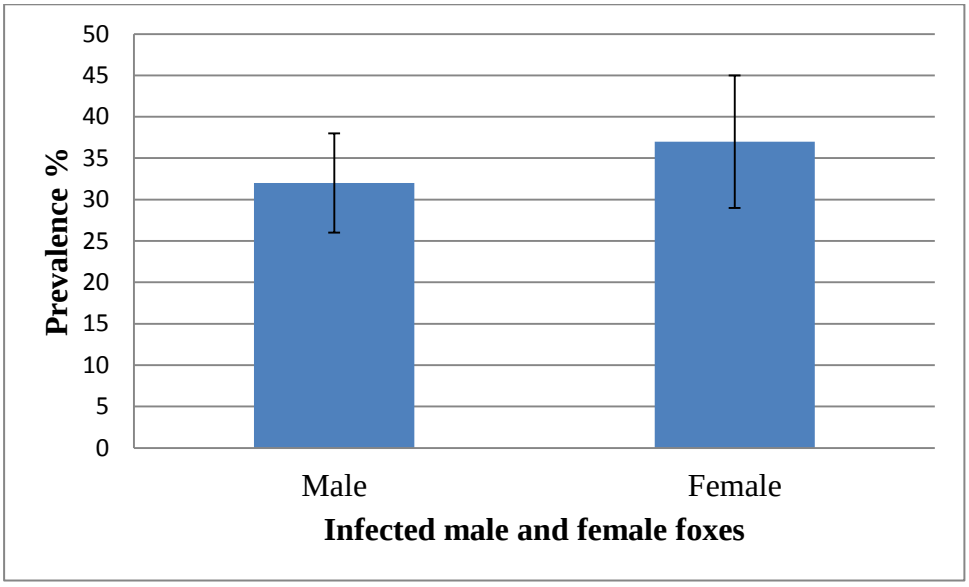


Figure 5.5. The prevalence of *Babesia* by gender of fox.

5.3.2.2. Infection of *Babesia vulpes* by seasonality.

The prevalence of infected foxes was 92/194 (47%, (95% CI 40.5-54.5)) in Autumn and 42/198 (21% (95% CI 15.9-27.3)) in Winter. There was a significant association between seasonality and probability of infection (χ^2=29.921, 1 d.f., P = 0.000) where foxes were more likely to be infected with *Babesia* in the Autumn than in Winter .

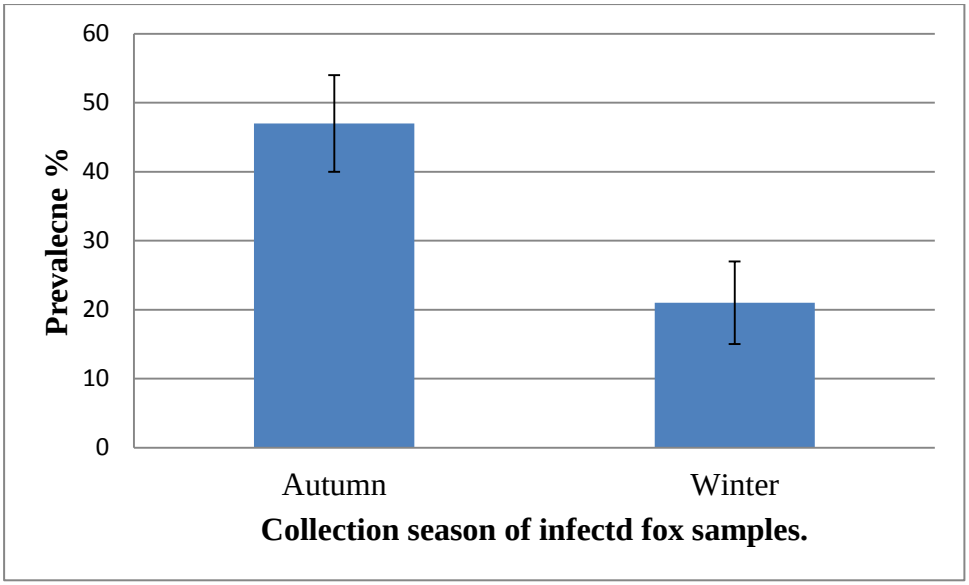


Figure 5.6. Prevalence of *Babesia* in red foxes over two seasons.

5.3.2.3. Infection of *Babesia vulpes* by different age groups.

With respect to age of foxes, the prevalence of infection showed some association with younger age; 101/275 young (36% (95% CI 31.2-42.5) were infected followed by those foxes considered to be aged at 32/112 (28% (95% CI 20.8-37.4)) and juvenile foxes at 1/5 (20% (CI 6-72%). However, there was no significant association between different age groups and probability of infection (χ^2=2.806 1 d.f., P = 0.246) (Figure 5.7).

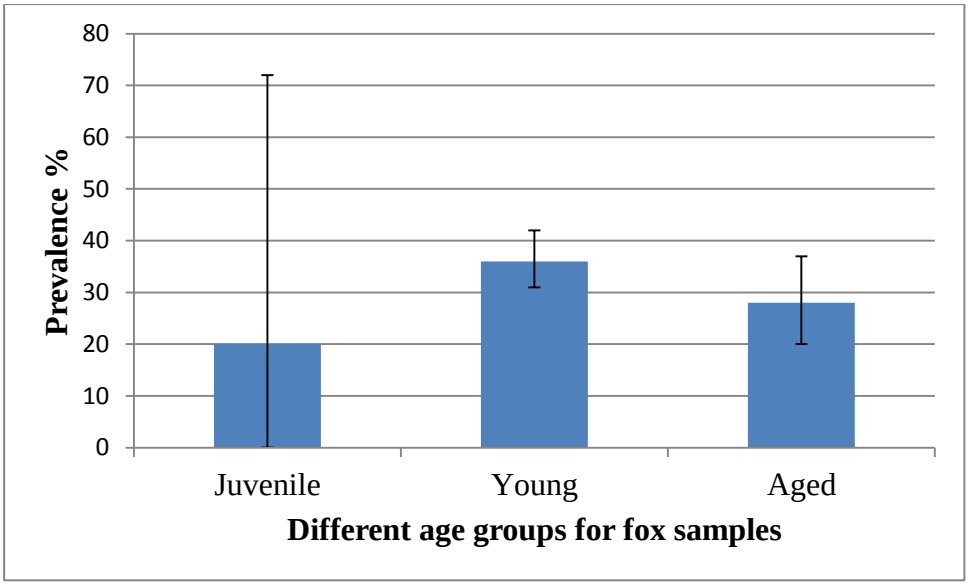


Figure 5.7. Prevalence of infection by age groups.

5.3.2.4. Infection of *Babesia vulpes* by different body condition.

The prevalence of *Babesia* infection was studied based on body condition. *Babesia* parasites were commonly found in foxes that had a fair body condition in 16/34 (47% (95%CI 31-63%) animals followed by 116/348 (33% (95%CI 29-38%) and 2/10 (20% (95%CI 6-51%) for good and poor body condition, respectively. There was no significant association between different body status and probability of infection (χ^2=3.511, 1 d.f., P = 0.173) (Figure 5.8).

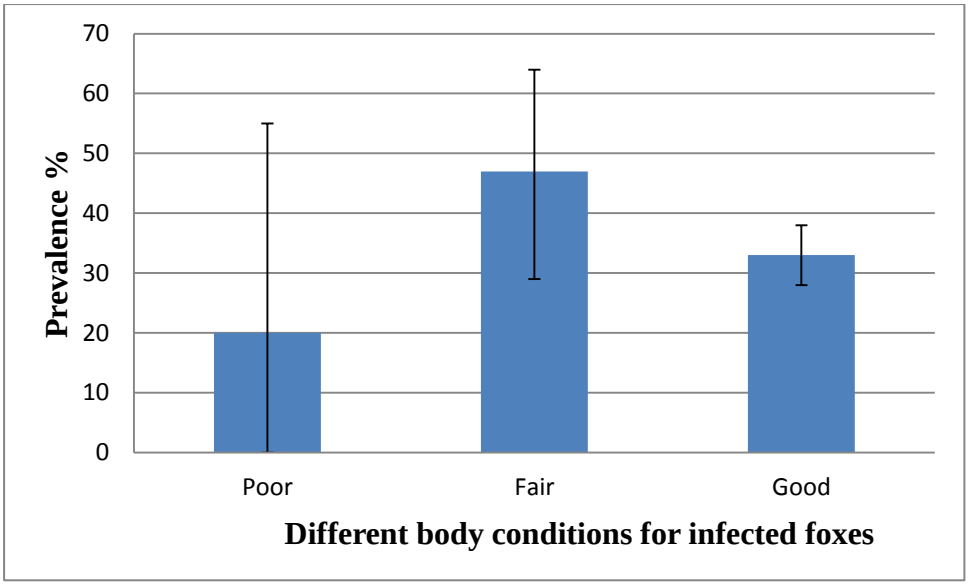


Figure 5.8. Prevalence of *Babesia* in foxes based on body condition.

5.4. Discussion.

In this study, the overall prevalence of *Babesia vulpes* from red foxes in the UK was 34%, which suggests that the parasite is potentially widely distributed among the red fox population throughout south-west England. Prevalence of the infection in this study demonstrated that females (37%) were more likely to be infected than males (32%), although these difference were not statistically significant (P<0.292). This is likely related to the small size of the sample and efforts to include additional samples may see statistical significance emerge for this variable. Previous prevalence studies of parasitic infection of mammalian hosts have shown that females are more likely to be infected than their male counterparts and this may be because of the different levels of hormone and innate immune response (Aguilar-Delfin *et al.*, 2001; Roberts *et al.*, 2001). However, the difference between male and female foxes regarding *Babesia vulpes* is not fully understood and there are some studies, which suggest that males may more likely to be infected with parasite species (Macnab *et al.*, 2016; O'Donnell & Beshers, 2004). In addition, a prevalence study of *Angiostrongylus* and *Eucolius* in red foxes in England showed no difference by sex and infection with these parasites (Morgan *et al.*, 2008). Furthermore, Hodžić and colleagues (2015) did demonstrate that no significant difference between sex or age of red foxes and infection with *B. vulpes*. However, females were more likely to be infected by *Hepatzoon canis* than males.

It should be noted however that in a study by Bartley and colleagues, male foxes were found to have a higher prevalence of *B. vulpes* DNA than females in all regions of Britain (Bartley *et al.*, 2016). Thus, while our study adds to the evidence base, future studies are warranted to fully elucidate the sex dominance in relation to *Babesia*.

This study reports a higher prevalence of infection in the younger population (36%) while 32 out of 112 (28%) of the adult group were infected. The juvenile population had the lowest prevalence of the infection 20% (1/5) although sample size was again small for this group. However, these results illustrate no significant difference was reported thus the lack of significant differences in *Babesia vulpes* infection rates between different age class suggest age related differences might not influence the establishment of the infection in fox population (Cardoso *et al.*, 2013).A prevalence study of canine babesiosis in dogs in France illustrated that sex and age do not have any influence on the host susceptibility to the infection (Martinod *et al.*, 1986).

Furthermore, the body condition of each fox was recorded and used for further analysis, with high prevalence of 47% (16/34) reported in foxes with a fair body condition, followed by 33% (116/348) in those with a good body condition and 20 % (2/10) of foxes with poor body condition. In fact there was no significant difference between those groups either but this could be due to the small sample size of the foxes with poor body condition.

Interestingly, this study found that the infection rate for red foxes in Autumn was 47% (92/194) which dropped to 21% in Winter (42/198) and this difference was significantly different. This study had access only to samples collected in the Autumn and Winter. The researcher attributes this change in the rate of infection to the tick vector being active in the UK between Spring, Summer, and Autumn rather than in Winter (Abdullah *et al.*, 2016). These findings are supported by Najm et al 2014 study in Germany, which concluded that red fox infection rates were higher in June and October, and it has been recorded that ticks were often positive in October. However, we must acknowledge that the sampling of red foxes only occurred due to premature death of foxes through road traffic trauma. Thus, it might be that this our study provides an under-estimate of the parasite prevalence of red foxes in Winter because they are more likely to hibernate at this time.

The observed prevalence of the infection in this study was higher than that previously reported in other studies in Hungary (20%) (Farkas *et al.*, 2015) , Italy (0.98%) (Zanet *et al.*, 2014), and an older UK study (14.4%) (Bartley *et al.*, 2016), while the highest prevalence was detected in foxes from Portugal (69%) (Cardoso *et al.*, 2013), Austria (50%) (Duscher *et al.*, 2014) and the USA (39%) (Birkenheuer *et al.*, 2010). The different prevalence rate of the infection in this study and previous studies can be attributed to different extrinsic factors such as type of tissues sampled, number of samples, detection assay methods, how samples were collected (road traffic accident) and probably vector distribution.

To our knowledge, this study is one of the first studies to investigate the presence of *Babesia vulpes* DNA in the UK fox population using the amplification of beta-tubulin following the study by Bartley, which detected the presence of *Babesia vulpes* in the UK fox population using 18S rRNA. Our phylogenetic analysis demonstrates that the beta tubulin gene of *Babesia vulpes* generated in this study shares ≥98% sequence identity with the Massachusetts fox which has been recorded in GenBank under accession number

AY17709. This also shared ≥92% homology with beta tubulin gene described from a Spanish dog, under accession number AY144709.

The presence of *trypanosoma* infection among red fox populations have been investigated by some of studies, which showed that no red foxes were infected with *trypanosoma*, which supports the finding in this study (Rosypal *et al.*, 2014). However, *T. cruzi* was reported in grey foxes from North Carolina, Virginia, Pennsylvania and Tennessee (Rosypal *et al.*, 2010, , 2014). It is highly likely that our results can be attributed to the absence of the competent vector (triatomine bug) for *T. cruzi* in the United Kingdom.

Although there are few studies which have suggested that red foxes can be reservoir host for different vector-borne bacteria such as *B. rochalimae* that reported in France, USA and Iraq (Chomel *et al.*, 2012; Halliday *et al.*, 2015; Henn *et al.*, 2009), the presence of this pathogen cannot be confirmed in this study, and similar results were been observed in red fox population from Bosnia and Herzegovina, where 119 of red fox had negative results for *Anaplasma* and *Bartonella* (Hodžić *et al.*, 2015). The negative results from our study can be due to different PCR assay and primers which had been used. Interestingly, 15 out of 39 from red foxes in Iraq were positive for *Bartonella* species using an antibody-antigen assay and none of those samples were positive when tested by PCR (Chomel *et al.*, 2012). Thus , the the positive results can be due to the previous infection with *Bartonella* spp.

The absence of other haemoparasite species such as *Trypanosoma* and *Bartonella* in this study might be due to the low level of parasitaemia in the blood or the low prevalence of infections in animals from the collected area. Without performing a larger, cross-sectional across the UK by seasonality, it is very difficult to truly confirm the absence of these species but given the absence of such infections clinically in mammals or humans in the UK, there appears to be no public health issue associated with these reservoirs with respect to these particular pathogens.

Chapter Six

Comparison of molecular methods to investigate tick borne infections: Next Generation Sequencing (NGS) vs. Polymerase chain reaction.

6.1. Introduction.

Tick species are important vectors for different diseases such as lyme borreliosis and babesiosis which can infect and cause illnesses in animal and human populations around the world (Kasozi *et al.*, 2014; Schwameis *et al.*, 2017). They are considered to be the second most important vector of infectious diseases after mosquitoes (Hillyard, 1996), which they spread through attaching to their host and transmitting the pathogen when taking a blood meal. For example, the incidence of human borreliosis in the United Kingdom has increased 3.6 fold since 2001, with more than 950 cases that were reported in 2011 by the Health protection agency (Dubrey *et al.*, 2014). Ticks can spread to different geographical regions by colonizing traveling pets and by other types of transportation (Otranto *et al.*, 2009). Thus, many infections have spread to different parts of the world. For example, the presences of tick borne encephalitis have been reported in 27 European countries and in some Asian countries (Amicizia ,2013). Furthermore, human incidence of these infections have been reported in Russia and other parts of Europe at 10000 and 3000 clinical cases, respectively (Charrel *et al.*, 2004; Heyman *et al.*, 2010; Mansfield *et al.*, 2009). The transmission cycles of tick borne disease are maintained through a complex cycle, which involves several host reservoirs. To have a more complete understanding of how parasites circulate, the interaction between the vector and the host and the disease risk in the area must be examined.

The detection of different pathogens from tick samples has been investigated by different techniques such as microscopy, culture, serology, polymerase chain reaction (PCR), real-time PCR and reverse line blot (RLB) hybridisation. Microscopic examination of blood smears was traditionally used to detect different tick borne disease. With respect to *Anaplasma* spp., microscopic examination was the most common detection tool in cattle populations using blood smears (Ybanez *et al.*, 2013). The sensitivity of this technique has been shown to be 10^6 in infected red blood cells per millilitre (Gale, 1996). However, due to the low level of parasitaemia in infected hosts and difficulty distinguishing between *Anaplasma* and other structures, this tool has not been recommended for the characterization of infection in infected cattle (Carelli *et al.*, 2007), while molecular techniques appear to offer improved sensitivity and specificity. While culture of pathogens has been used in diagnosis, it is not a routinely available diagnostic tool for the diagnosis of lyme borreliosis in clinical practice, due to many factors such as low sensitivity, long incubation period, and the requirement of special media and expertise (Marques 2015).

Indeed, specialist reference laboratories are often required for detection of *Borrelia* organisms due to the difficulty in identifying 'active' infections (Mavin *et al.*, 2015). Serology methods provide the basic requirements for the diagnosis of tick borne diseases and have been used to detect *Babesia divergens* in cattle populations (Zintl *et al.*, 2003). However, traditional double-tier (enzyme immunoassay [EIA] screening and Western blot confirmation) testing for the laboratory diagnosis of Lyme borreliosis (LB) is expensive, lacks sensitivity in the diagnosis of early borreliosis, does not distinguish between active and past infection and cannot be used as a marker for treatment response (Mavin *et al.*, 2014). Conventional PCR has brought many benefits including quick diagnosis for new infections and it can identify and characterize new species of tick borne pathogens. Furthermore, it helps biological scientists to understand the DNA sequences, gene expression, and genetic analysis. These provide a better understanding of organisms at the DNA, RNA, and transcriptome levels (Speers *et al.*, 2003). The technique has been used by many laboratory studies to investigate the presences of tick borne parasites such as *Babesia* and *Theileria* spp. in livestock species around the world (Oliveira *et al.*, 1995; El-Ashker *et al.*, 2015; Pienaar *et al.*, 2011). Real-time PCR has been used in many studies and in clinical diagnostics due to its ability to generate quantitative results, which can be obtained in a short timeframe and accurate fashion compared with conventional PCR that generally gives qualitative results (Kubista *et al.*, 2006; Morillo *et al.*, 2003). This technique has been used been for the detection of a number of bacteria including *Borrelia*, *Rickettsia,* and *Anaplasma* spp (Kato *et al.*, 2013; Ramos *et al.*, 2014; Schlachter *et al.*, 2017) throughout different regions around the world.

Although these techniques have been used to detect different infectious diseases, there are some technological limitations of the aforementioned methodologies. For example, the use of specific primers in conventional molecular approaches (PCR) is only able to detect specific organisms so novel infections may potentially be missed. Furthermore, the culture–based methods also suffer from the same limitations as isolation of cells from culture medium and particular pathogens can be very difficult to identify (Vayssier-Taussat *et al.*, 2013). However, the recent development of next generation sequencing (NGS) methods and bioinformatics analysis has undoubtedly had a significant impact on the ability to detect several infectious diseases, while also having much more discriminatory capabilities (Sabat *et al.*, 2017).

DNA sequencing is a method of determining the exact order of nucleotides within a DNA strand. DNA sequencing has become very important for most branches related to the life sciences. Sanger sequencing is considered as the first generation sequencing process, which was developed in 1977 by Sanger (Sanger *et al.*, 1977), his methods were to become the fundamental cornerstone for DNA sequencing for many years. The first generation sequencing method was used to undertake the Human Genome Project (HGP) which took approximately 13 years to be elucidated and it was completed in 2003. However, due to some inherent limitations including speed, resolution and throughput of first generation sequencing projects, next generation sequencing (NGS) was developed (Shin *et al.*, 2014). NGS enables inexpensive (per base), rapid, parallel sequencing of entire eukaryotic genomes, while alterations to its methodology can also allow sequencing resources to be focused on single or multiple informative loci. The utility of NGS is considered to be a massive parallel of deep sequencing technology, which increases our ability to understand and characterize tick borne disease. It has become possible by using next generation sequences to have rapid DNA sequences directly from the competent vector and generate a wide overview of microbial communities with this vector. Through targeting different parts of the pathogen genomes. NGS can provide identification of parasites community at deferent taxonomical level such strain and family (Kuleš *et al.*, 2017). NGS has been used by some laboratory studies to identify several microbial pathogen in tick species by targeting the 16SrRNA gene. Furthermore, it also has been used in animal tissue samples to detect their pathogen (Wittekindt *et al.*, 2010). The recent development of NGS allows many researchers to investigate different tick borne diseases using such a technique. For example, the technique was able to detect different species of parasites including *Babesia* and *Theileria* spp from *Ixodes* ticks in France (Bonnet *et al.*, 2014). In addition, different bacterial infections including *Borrelia, Rickettsia* and *Candidatus Neoehrlichia* have been found in *I. ricinus* using NGS as a detection tool (Carpi *et al.*, 2011).

Furthermore, the ability to analyse microbial communities without culturing organisms has resulted in new technologies such as the increasing field of metagenomic and microbiome analysis, which has generated significant new insights into the relationship between host and microbe (Goldberg *et al.*, 2015). Such techniques have been used to demonstrate mutual genetic pathways shared by tick borne pathogens, which have co-evolved over time (Lockwood *et al.*, 2016).

Aims

The aim of this chapter is to assess the use of NGS and other PCR assays by investigating the prevalence of different infection in tick samples, which were collected from Woodchester Park, England.

The detailed objectives are set out as follows:

- To investigate the prevalence of Haemoparasite infection in ticks using a nested-PCR as a diagnostic tool.
    - Investigation of other microorganism using the 16S rRNA gene using Next-Generation Sequencing methods, conventional and real-time PCR.
    - Compare sensitivity of the methods.

6.2. Methods.

A total of 40 tick samples were collected from Woodchester Park in 2017. All collected ticks were identified as *I. ricinus* and they were at the nymph stage. The DNA was extracted as it been described in 2.4.1. The detection of different parasitic infections (*Trypanosoma,Babesia, Theileria* and *Bartonella*) was performed by using a nested PCR assay that described in chapter 2.3.2, which includes two rounds of PCR with four sets of specific infection primers that amplified the 18S rRNA gene for *Trypanosoma, Theileria, Babesia* and the *gltA* gene for *Bartonella*. Following the previous step, each of the PCR products were visualized by using gel electrophoresis in order to identify the correct band size(chapter 2.4.).

Next generation sequencing was applied to the forty samples and investigation of 16S rRNA infections has been done using specific PCR conditions that were described in chapter 2.8.2. After the first PCR, the samples were purified as previously described in section 2.8.3. This was followed by a second PCR (index PCR) that was described in 2.8.4. Again, a second clean up was done for all samples as described in section 2.8.5. Next, DNA concentration and quantification were done as described in sections 2.8.6. The following step was to prepare the samples for loading into the machine as was previously described in section 2.8.7**.**The detected species by NGS was confirmed using real-time PCR (for *Anaplasma* spp, *Borrelia* spp, and *Rickettsia* spp) as it was described in 2.3.3 and conventional PCR (for *Candidatus Midichloria* spp) in 2.3.2.

6.3. Results.

6.3.1. Investigating the presence of haemoparasites using nested PCR.

The results in this study illustrated that none of the 40 ticks were infected with *Babesia, Trypanosoma, Theileria* and *Bartonella,* which is reassuring from a public health perspective. However, this was a very small sample size therefore it is difficult to extrapolate the findings beyond that of this study.

6.3.2. Investigation the presence of different bacteria species using 16SrRNA by NGS.

NGS was used to investigate the presence of bacteria species by amplifying the 16S rRNA. Of those samples, 25 out of 40 were positive for different species of pathogens. The species of bacteria which were detected were *Candidatus Midichloria, Borrelia, Anaplasma,* and *Rickettsia.* A high prevalence of *Candidatus Midchloria* was noted (23/40), followed by *Rickettsia* (4/40), *Borrelia* (3/40) and *Anaplasma* (1/40). Due to the short read sequencing of a single variable region (in this case V4) we were not able to confidently classify the data to the species level, but the data were classified to the genus level (Figure 6.1). The obtained sequences of *Borrelia spp, Rickettsia* spp and *Anaplasma,* in this study illustrated high similar (99%) to *B. garinii* (KY312118.1), *R.helvetica* (KU310588.1) and *A. phagocytophilum* (LC334014.1) that are deposited in Genbank.

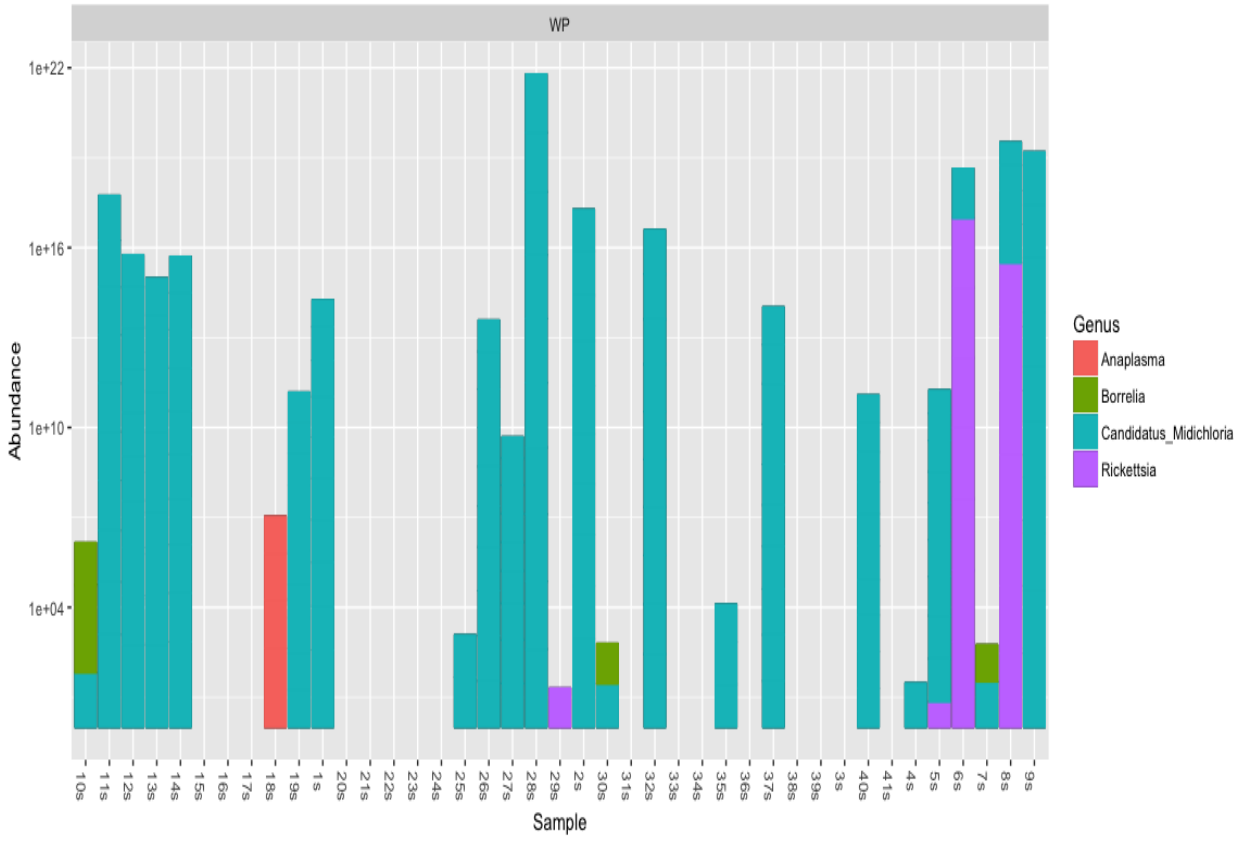


Figure 6.1.Genus level confirmation of pathogens detected from *Ixodes* ticks samples (40samples) in Woodchester Park, UK.

The presence of these bacteria was investigated using real-time and conventional PCR in order to combine the obtained results of NGS and those techniques. There was some variation between the two techniques in this study, where both techniques (NGS and Real time PCR) had the ability to detect the same number of positive samples (same ID) for *Anaplasma phagoctophilum* and *Rickettsia helvetica* infection. The variation in the number of positive samples was observed for *Borrelia garinii* and *Candidatus Midchloria*. Tick samples were more likely to be positive for *Borrelia* infection using the real-time PCR technique than the NGS technique, for example the three positive samples which were detected by NGS were also detected by real- time PCR plus two additional samples. However, this difference was not significant (Fishers Exact test, P=0.65). The presence of *Candidatus Midichloria* infection was detected in 23 samples using NGS and in 20 samples (three samples were negative out of 23) using conventional PCR (Table 6.1), although again this difference in sensitivity was not significant (χ^2=0.556, 1d.f., P=0.46).

Table 6.1. The prevalence of haemoparasitic infections in *I. ricinus* (n=40) detected by NGS and real time PCR, while *Candidatus Midichloria* samples were detected by PCR.

Pathogen	**Positive NGS/prevalence**	**ID number**	**Positive PCR/prevalence**	**ID number**
Anaplasma phagocytophilum	1 (2.5%)	18s	1 (2.5%)	18s
Borrelia garinii	3 (7.5%)	7s,10s and 30s	5 (12.5%) (real-time PCR)	7s, 8s,10s,30s and 32s
Candidatus Midichloria	23 (57.5%)	1s,2s,4s,5s,6s,7s,8,9s, 10s,11s,12s,13s,14s,19 s,25s,26s,27s,28s30s,3 2s,35s,37s and 40s.	20 (50%) (conventional PCR)	1s, 2s, 5s, 6s, 8s, 9s,10s 11s,12s,13s,14s,19s,25s ,26s,27s,28s,32s,35s, 37s and 40s.
Rickettsia Helvetica	4 (10%)	5s,6s,7s and 29s	4 (10%)	5s, 6s, 7s and 29s

6.4. Discussion.

Real time PCR assays and NGS have emerged as techniques that could be the gold standard for detection of different parasites and pathogens of public health importance. The presence of different haemoparasitic infections including *Trypanosoma, Babesia, Theileria* and *Bartonella* were investigated in this study using a number of different PCR assays. Furthermore, in this study, all 40 tick samples were free from any haemoparasites including *Babesia*, *Trypanosoma, Thieleria*, and *Bartonella*.

Samples were screened for the presence of a variety of bacteria using the NGS and PCR approaches, among the Anaplasmataceae family, one sample was identified as being *A. phagocytophilum*, the agent of human granulocytic anaplasmosis The prevalence of the pathogen in *I. ricinus* species has been reported in different parts of Europe such as France, Austria and Swaziland at 0.7,1% , and 1.4%, respectively (Glatz *et al.*, 2014; Oechslin *et al.*, 2017; Reis *et al.*, 2011).When the techniques of NGS and PCR were used followed by DNA sequencing approaches. The results in this study were in agreement with a previous study by Bown 2006 who detected the same pathogen in 11 /163 (6.7%) field voles (*Microtus agrestis*) and in three samples of *I. ricinus* (2 larvae and one nymph) collected from field voles in Northern England (Bown *et al.*, 2006). Furthermore, in 2012, Taussat and colleagues isolated bacteria from bank voles in France (Vayssier-Taussat *et al.*, 2012).

Lyme borreliosis is regarded as the most common tick-borne infection of humans in temperate parts of the world (Parola & Raoult, 2001). In this study, the presence of *Borrelia spp* DNA was detected in five samples (three samples were detected by NGS (samples ID number 7s, 10s and 30s) and five samples (samples ID number 7s, 8s, 10s, 30s and 32s) by real-time PCR) and *B. garinii* was identified as being the species.

Another bacterial organism that we identified was *Candidatus Midchloria*, which is an intracelluar bacterium that can invade and destroy the mitochondria of *I. ricinus*. This bacterium derives from a lineage with the *Rickettsia* (*Alphaproteobacteria*) as reported by different studies (Sassera *et al.*, 2006). Using different approaches of NGS and PCR, 23 ticks were reported to be infected with this bacterium using the NGS technique, whereas only 20 (Table 6.1) ticks were confirmed when using the PCR approach. The last bacterial infection that was reported in this study was *R.helvetica*. Using both techniques of NGS

and real tim PCR, we were able to detect 4 infected ticks (with same ID number, Table 6.1) in this study.

The difference between the two approaches was in the ability to detect *Candidatus Midchloria* and *Borrelia garinii*. For *Candidatus Midchloria*, 23 positive ticks were confirmed using the NGS approach while only 20 samples were confirmed using PCR, and this results indicates that NGS was more sensitive than normal PCR. Similar results were obtained in Swaziland, where eight samples of *I. ricinus* were positive for *Candidatus Midchloria* using NGA and none of these samples were positive by conventional PCR (Oechslin *et al.*, 2017).With regard to *Borrelia garinii* infection, NGS was able detect 3 positive samples whereas real time PCR was able to confirm 5 samples as being infected with *B. garinii* (Table 6.1). The explanation for the two extra samples (8s and 32s) in qPCR can be due to the high sensitivity for the qPCR in detection the low number of pathogens within those samples since the amplification was very late (after 35 cycle), and it can be attributed to using specific probe that able to detect the target gene, which may had less number of reads in the NGS. Furthermore, it can be due to the use of the 16S primer that may has less sensitivity for broad range amplification of low copy number pathogens.

Few studies have compared the new NGS approach with different approaches such as qPCR in detecting tick- borne diseases. The study by Bonnet et al, (2014), showed the powerful utility of using NGS in detecting *Babesia* parasites in tick samples. The techniques were able to confirm different species of *Babesia* such as *B. microti*, *B. divergens*, *B. major*, and *Babesia* sp. EU1 (Bonnet *et al.*, 2014). When comparing NGS with qPCR, the only Babesia species that was detectable by qPCR was *Babesia* sp. EU1. While the techniques were not able to detect any of the other three species that were confirmed by NGS, This results can be due to the small number of the parasites in those samples, which is under the PCR threshold but they were detected by NGS due to the large number of transcripts of the 18S rRNA gene, NGS showed the powerful utility of using this new technique in screening for tick- borne diseases (Bonnet *et al.*, 2014).

In Western Europe, bacterial pathogens were investigated in tick species using NGS and the results were confirmed by qPCR, NGS were able to detect diferent species of bacteria including *Anaplasma* spp, *Ehrlichia* spp, *Francisella* spp, and *Rickettsia* spp. When

comparing NGS and qPCR, the presence of *Anaplasma* spp. DNA was the only species that was confirmed by qPCR (Vayssier-Taussat *et al.*, 2013).

There are several limitations to our PCR assays and NGS comparative study. We only performed this prevalence study on 40 ticks, which is a very small sample size. Although all 40 tick samples were free from any haemoparasties including *Babesia, Trypanosoma, Thieleria,* and *Bartonella*, we were able to identify several organisms of public health importance including *Borrelia garinii, Rickettisa helvetica* and *A. phagocytophilum* by using NGS. A recent prevalence study of cats in Great Britain found that the prevalence of ticks on cats approximated 7%, and of those colonized, 1% of those ticks were infected with both *Babesia* and *Borrelia garinii* (Davies *et al.*, 2017). Furthermore, we also detected spotted fever group (SFG) rickettsiae in several ticks in this study, which corroborate previous studies by Tijsse-Klasen (2013). We also identified the endosymbiont *Candidatus Midichloria* from ticks, which has been shown before thus our methodology was consistent with other studies (Smith & Wall, 2013). In this study, the NGS has been able to detect different bacterial infection from *Ixodes* communities as other studies.

Tick borne diseases have been considered a major public health problem for humans, while they also cause considerable economic issues in wild and domestic animals around the world. This study evaluated the potential of different molecular techniques including PCR, qPCR and next generation sequencing (NGS) to detect different bacterial infections that are carried by *I. ricinus* from the United Kingdom. Sequences corresponding to different bacterial species included *Borrelia garinii, Anaplsma phagocytophilum, Rickettisa helvetica* and *Candidatus Midichloria.* These pathogens were detected by all techniques with some variation observed regarding the positivity of certain pathogens. The results obtained from this study have demonstrated that NGS has huge potential to detect different number of bacterial infections, which have been reported previously by many studies in the United Kingdom. For future studies, the use of NGS would be useful in investigating the presences of parasitic infections using 18S rRNA (Bonnet *et al.*, 2014). This will help many researchers to understand the transmission pattern of these parasites within tick populations in certain environments. Furthermore, such discriminatory methods may be able to help identify *Babesia* species, some of which may be zoonotic, as was identified in a recent French study by Bonnet and colleagues (2014). This was the first time that *B. microtii* and *B. major* had been identified in France. This approach also

identified new sequences corresponding to as-yet unknown organisms similar to tropical *Theileria* spp. (Bonnet *et al.*, 2014).

Chapter Seven

General discussion.

7. Discussion.

The overall aim of this study was to use molecular methods to investigate vector-borne diseases from various hosts and locations to increase our understanding of the factors that may affect their distribution.Vector-borne diseases (VBD) have gained global recognition as posing various risks to animal and human health. Arthropods such as sandflies, ticks and mosquitoes facilitate the transmission of protozoa, viruses and bacteria that cause fatal illnesses such as Crimean-Congo hemorrhagic fever, dengue fever and malaria (Maillard & Gonzalez, 2006). It has long been established that diseases such as malaria and leishmaniasis remain prevalent throughout various countries across the world where they result in significant morbidity and mortality (Hotez *et al.*, 2006).

Harrus and Baneth illustrated that VBD have re-emerged in many parts of the world and they cause health risks to wildlife, domestic animals, livestock and human populations (Harrus & Baneth, 2005). Studies of arthropod-borne infections in wildlife species can provide insights to infections of human and animals. Furthermore illnesses that emerge from wildlife species have acquired extensive recognition in the past when they were identified as posing risks to agricultural systems and to humans.

In this study, the main aim is to investigate haemoparasite infections from different host and vector species that were sampled from the United Kingdom and Saudi Arabia. One innovative aspect in this study is the attempt to ascertain the prevalence and genetic diversity of haemoparasites from two different parts of the world, which differ by climate, rurality and vectors. Nonetheless, the technology to detect the parasites remains the same. The chosen haemoparasites were *Bartonella*, *Babesia*, *Theileria*, and *Trypanosoma* spp, while the animal hosts that have been examined included rodents , foxes and tick in the UK ,rodent and shrew in Ireland and hedgehogs and jird from Saudi Arabia.

It is clear from this study that the number of potentially important VBD's is likely to increase as many are still to be identified. Wildlife in many areas of the world has yet to be investigated as potential reservoir hosts for haemoparasites. In Chapter 4 this study reports novel species of *Bartonella* and *Theileria* in both rodents (Libyan Jirds) and insectivores (Desert hedgehogs). What is not known is whether any of these novel species has the potential to infect humans or domesticated animals. Previous studies of *Bartonella* spp of rodent origin have identified them as potential human pathogens. For example, *B.elizabethae* has been identified in many wild animals, particularly rats, and it has been

implicated as a potential pathogen that can infect humans around the world (Chomel, 2006). This study has demonstrated that different *Bartonella* spp were variable between both jirds and hedgehogs from Saudi Arabia. The Saudi samples formed 3 clades in the phylogenetic analysis with *Bartonella* species that were previously reported in Genbank. Furthermore, jird isolates which included sample sets 10, 86 and 1 were very similar to *B. elizabethae* with (97%), *B. grahamii* (92%) and *B. rochailime* (98%) similarity, respectively. These study outcomes indicate significant public health importance as a jirds in Saudi Arabia were frequently infected with *Bartonella* strains that are closely related to organisms identified as human pathogens. The potential for humans acquiring the infection in Saudi Arabia is not very clear but it should be noted that *B. elizabethae* has been isolated from several febrile patients in Thailand, Indonesia and the United States, presumably again through zoonotic infection (Colton *et al.*, 2010; Eremeeva *et al.*, 2007; Oksi *et al.*, 2013; Winoto *et al.*, 2005). Consequently, our research warrants further investigation on other reported species of *Bartonella spp* in Saudi Arabia.

The presence of *Theileria DNA* was reported in Libyan jirds (40%) and desert hedgehogs (66%) from Saudi Arabia samples. To my knowledge, this study is one of the first to consider detection of the presence of these parasites in these animal species in Saudi Arabia. Again, the high prevalence of infections is of particular concern as previously *Theileria* species from hedgehogs have been associated with disease in humans. A study in China reported that hedgehogs were infected with *T. lunwenshi* spp. and *Theileria* spp. that was isolated from a febrile hospitalized patient (Chen *et al.*, 2014). Although this appears to be a sporadic report, it does suggest that there is a potential concern that hedgehogs could act as a reservoir host and contribute to zoonotic transmission of the disease. Given that *Borrelia miyamotoi, A. phagocytophilum, R. helvetica* and *B. burgdorferi* genospecies (*Borrelia afzelii, Borrelia bavariensis* and *Borrelia spielmanii*) were all detected from both *I. hexagonus* and *I. ricinus* ticks that were feeding on European hedgehogs, zoonotic transmission is perfectly plausible (Jahfari *et al.*, 2017).

More work needs to be done in Saudi Arabia. For example, screening more areas will increased our understanding about what potential of threaten pathogens present in these areas. More generally, this highlights that in many areas little is known about parasites circulating in wildlife and their potential threat to humans and domesticated animals.

Even in locations where extensive studies of wildlife and VBD's have been undertaken, many species have not been intensively sampled, perhaps because of difficulties in collection suitable samples. In the UK for example, many studies have focused on easily sampled species such as small mammals, whilst even abundant species such as foxes have been relatively poorly studied. This study took advantage of samples collected in an opportunistic manner to investigate VBD's in foxes. Red foxes have been reported as important host reservoir for some zoonotic infections such *as Toxoplasma gondii* and *Bartonella* spp (Dubey *et al.*, 2014; Kaewmongkol *et al.*, 2011) around the world. In the UK red foxes are very abundant with an estimated population of between 240,000 to 258,000 (Bartley et al., 2016). In the United Kingdom little is known about the different pathogens that infect fox population and could have significant impacts on other animal species. For example, the recent detection of *Babesia vulpes* in fox population has a significant impact of the dog population (Zahler *et al.*, 2000).This study was able to detect *Babesia vulpes* from red foxes within South-west England . DNA sequences of *Babesia* had 98% homology to *Babesia microti* isolate SN87-1 beta n-tubulin gene (isolated from a fox in the USA) on the NCBI database (AY144707.1). Approximately 34% of foxes were infected with *Babesia vulpes*.

The parasite has been reported from different parts of the world among red foxes population .In this study the prevalence of the infection was higher than that previously observed in other studies in Hungary (20%) (Farkas *et al.*, 2015), Italy (0.98%) (Zanet *et al.*, 2014)and previous UK study (14.4%) (Bartley *et al.*, 2016). The highest prevalence was detected in foxes from Portugal (69%) (Cardoso *et al.*, 2013) , Austria (50%) (Duscher *et al.*, 2014) and the USA (39%) (Birkenheuer *et al.*, 2010).

Different factors may affect the prevalence of *Babesia* infection such as season of collection and vector distribution. For example, in Germany, the prevalence of *B. vulpes* in red foxes was higher in Autumn (specifically in October) and this was attributed to vector activity which been more active during this month (Najm *et al.*, 2014). Interestingly, the results in this study was similar to a previous study in Germany, where foxes were more likely to be more infected in Autumn than Winter ($P<0.05$).

The presence of *B. vulpes* in the United Kingdom has been recently investigated by amplification of the 18srRNS gene in 2016. The results confirmed the presences of *B. vulpes* among red foxes and has been reported in several laboratory studies around the

world (Bartley *et al.*, 2016; Birkenheuer *et al.*, 2010; Cardoso *et al.*, 2013). This study is considered to be one of the first studies to investigate the presence of *B. vulpes* infection in the UK fox population using the amplification of beta-tubulin. Our phylogenetic analysis demonstrates that the beta tubulin gene of *B. vulpes* generated in this study shares ≥98% sequence identity with the Massachusetts fox, which has been recorded in GenBank under accession number AY17709. This also shared ≥92% homology with beta tubulin gene described from a Spanish dog, under accession number AY144709.

It has previously been demonstrated that humans can have a significant impact on the distribution of diseases. Historically plague has been spread throughout the globe as a consequence of humans travelling endemic areas to non-endemic areas (Wilson, 1995). More recently, the movement of animals has also enabled increased distributions of some infections. For example ,the deliberate release of myxoma virus has had a significant impact on rabbit populations in Australia and Europe (Best & Kerr, 2000; Kerr *et al.*, 2015). Sometimes changes in the distribution of a disease is a non-intentional product of human activity, as is seen in the case of squirrelpox in the UK (Darby *et al.*, 2014). However, human activity may also disrupt natural-host parasite systems to reduce the prevalence of infections, such as that previously reported by Telfer et al 2005 regarding the impacts of bank vole on the prevalence of *Bartonella* spp that found in wood mice in Ireland .This study shows that Trypanosomes appear to be affected in the same way (no trypanosome in bank voles in Ireland, no infection in wood mice where there are bank voles). In this study, two species of *Trypanosoma* were reported in bank vole and wood mice which were collected from England and Ireland. The total prevalence of the infection is 0.8 (4/473). The parasite was detected in only 1 sample from bank vole in England whereas three wood mice were infected in Ireland. The prevalence of the infection in this study was lower than previous studies of the same species. For example, a study by Noyes et al., 2002 was able to report 27% prevalence in the bank vole and the wood mice.

In England, bank voles and wood mice are considered as the native species that occurs in many areas and the *Trypanosoma* parasites have been reported in both species by different studies (Noyes et al., 2002). However in Ireland thebank vole is considered to be an introduced species whilst wood mice are native.

To my knowledge this study is considered as the first study that investigated the presences of *Trypanosma* spp in Irish bank voles. The tested Irish samples were collected from areas

where both rodent species exist in sympatry and areas beyond the bank vole invasion range where only wood mice exist. *Trypanosoma* spp. infections were only found in areas where only wood mice existed. None of the bank voles had *Trypanosoma* infection in Ireland. Furthermore, no infection was reported in wood mice in area where invaded by bank vole. However, it has been reported that bank vole species in Ireland play an important role in the prevalence of other parasites species that infect wood mice. A study by Telfer et al (2005) illustrated that bank voles affected the wood mice *Bartonella* Interaction particularly *Bartonella birtlesii* and *Bartonella taylorii* . The prevalence of the infection decreased significantly in areas that have high density of bank vole. Also it has been reported that the prevalence of fleas species increased in wood mice areas that in the absence of the bank voles (Telfer *et al.*, 2005).In the present study similar results were obtained since the *Trypanosoma* were isolated in wood mice that lives this area (The fourth area). This can be due to the high prevalence of competent vector (fleas) which does not have other chance to interact with other hosts.The DNA sequences demonstrated that the isolate *Tryapansoma* from the bank voles were identical to *T. evotomys* that deposited in the Genbank (AY043356.1) while the three detected samples from wood mice were infected by *T. grosi* with accession number (AB175624.1).

One limitation faced by scientists investigating VBD's in wildlife is in the use of molecular methods to identify novel potential pathogens. Traditional PCR based methods can only be used to investigate infections that have already been identified.NGS as used here enables us to investigate the full range of infections present in each samples. NGS has made a significant impact on the medical field of microbiology and infectious diseases since a whole genome sequence of a pathogen can be obtained in a relatively short timeframe (Gullapalli *et al.*, 2012).

In this study, tick borne pathogens were investigated from among 40 *I. ricinis* samples, which were collected from Woodchester Park, England. Different molecular approaches have been applied throughout the study. For example, nested PCR was used to investigate different haemoparasitic species by amplifying the 18S rRNA from *Trypanosoma*, *Babesia* and *Theileria*, while the gltA gene for *Bartonella* was examined. Furthermore, the prevalence of different bacterial infections was performed by amplification of 16Sr RNA using NGS, real time, and conventional PCR since parasite detection was rare and we wished to utilize this technology in a real-world setting.

Several species of zoonotic and non-zoonotic bacteria including *Borrelia* spp., *Anaplasma* spp., *Rickettsia* spp., and *Candidatus Midchloria* were recorded using NGS and other PCR techniques. Interestingly, we observed some variation in the number of positive samples between different detection tools. The NGS results illustrated a high prevalence of *Candidatus Midchloria* (23/40), followed by *Rickettsia spp.* (4/40), *Borrelia* spp. (3/40) and *Anaplasma* spp. (1/40). Slight variations in numbers of positives were found when using real-time and conventional PCR methods, with more *Borrelia* spp. detected but less *Candidatus* Midichloria.

The variations between the two techniques have previously been reported by some laboratory studies that investigated the microbial communities in tick species. For example, NGS was able to detect deferent species of bacteria including *Anaplasma* spp, *Ehrlichia* spp, *Francisella* spp, and *Rickettsia* spp in tick samples from Europe. When comparing NGS and qPCR, only *Anaplasma* spp DNA was detected using qPCR while other infections were not detected (Vayssier-Taussat *et al.*, 2013). The explanation of the low ability of Real time PCR to detect the presence of *Ehrlichia* spp, *Francisella* spp, and *Rickettsia* spp is that the pathogens exist at a sufficiently low number that they are under the PCR threshold. Furthermore, other variations were reported between NGS and conventional PCR. The NGS was able to confirm the presence of Candidatus *Midchloria* in 23 samples whereas the pathogen was reported in only 20 samples using the conventional PCR. It may be that if affordable, a combination of both techniques could be used.

Although we demonstrated some variations in the study between different detection methods, these methods are considered to be gold standard approaches that have been used by many laboratory studies in order to identify and characterize new pathogens around the world.

The variation between all these techniques needs further more investigation to assess the sensitivity of each technique to detect certain infections.

8. Future Direction.

Further investigations are required to identify and characterize *Bartonella* species that occur in Saudi Arabia by screening more animal species since this study was able to detect some zoonotic species from a small number of animal hosts. Also it is very important to investigate the role of arthropod species in the transmission of the pathogens. The rapid development of detection tools such as NGS will help many researchers to identify other pathogens, which have not been reported in Saudi Arabia. Furthermore, further investigations of Herpetosoma species in rodent populations from the United Kingdom and Ireland are required to evaluate the prevalence of *Trypanosoma* infection, although this study would suggest it is incredibly rare. In Ireland, it is very impotent to understand the role of invading species (bank vole) which affect the prevalence rate of the infection on the native species, since bank voles have been reported to have a significant effect on *Bartonella* and helminth interactions in wood mice. The recent detection of *Babesia vulpes* in fox populations warrants further attention since the parasites have been reported to have clinical symptoms in dog populations. Finally, it is very important to investigate the prevalence of *Babesia vulpes* and other zoonotic pathogens in *Ixodes* species, which have been reported as competent vectors in different parts of the world. A recent study of ticks inhabiting cats from the UK would suggest that this approach has public health merit (Davies *et al.*, 2017).

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